

16/PR TS

## SPECIFICATION

### NOVEL POLYPEPTIDE

#### TECHNICAL FIELD

The present invention relates to a novel polypeptide which binds to JNK3 which is an isozyme of c-Jun N-terminal kinase (JNK); a DNA encoding the polypeptide; a vector containing the DNA; a transformant transformed with the vector; and a method for producing the polypeptide.

Furthermore, the present invention relates to an antibody which recognizes the polypeptide; a microorganism, animal cell or animal which produces the antibody; a method of screening a compound having a JNK3 signal transduction inhibitory activity using the polypeptide, a part thereof or a microorganism, animal cell or the like expressing the polypeptide or a part thereof; and a method of screening a compound which regulates gene expression of the polypeptide using the cell.

#### BACKGROUND ART

The cascade by mitogen-activated protein kinase (MAPK) which has an important role as an intracellular signal transduction molecule is an intracellular signal

transduction pathway universally existing in eucaryotic organisms ranging from yeast to human.

In vertebral animals, three kinds of MAPK: extracellular signal-regulated kinase (ERK), p38 and JNK/SAPK (c-Jun amino-terminal kinase/stress-activated protein kinase), and a large number of their activating factors have been identified, and the presence of various functionally different MAPK pathways has been revealed.

It is considered that JNK, among MAPK, is activated by various extracellular stress factors, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 (IL-1), epidermal growth factor (EGF), endotoxic lipopolysaccharide (LPS), heat shock, ultraviolet light (UV), X-ray and the like, and apoptosis (cell death) is induced by its activation (*Science*, 270: 1326 (1995)).

That is, in cells exposed to stress, when the stress signal is transferred to small G-protein (such as Rho, Ras or the like), MEKK1 (MAPK kinase kinase 1) as a member of MAPK kinase kinase (MAPKKK) is activated, and the activated MEKK1 phosphorylates SEK1 (also called MKK4) as a member of MAPK kinase (MAPKK) to activate SEK1. The activated SEK1 phosphorylates JNK (MAPK), and the JNK is activated. It is believed that the activated JNK phosphorylates c-Jun which is one of the transcription factors in the cell nucleus, and the transcription activity of the phosphorylated c-Jun is accelerated, then apoptosis

is induced in participation with AP-1, activating transcription factor 2 (ATF2) and the like. However, the process of this apoptosis in the nucleus is not well understood.

Apoptosis is induced when a neuronal cell, PC12, is cultured using a medium containing a nerve growth factor (NGF) as a proteinous nutrient factor of neuronal cells, and culturing is continued after removing NGF. It is known that increase in JNK activity is observed in apoptosis induction (*Science*, 270: 1326 (1995)).

It is known that, among three kinds of JNK (JNK1, JNK2, JNK3), JNK3 is expressed at particularly high level in the brain (*EMBO J.*, 15: 2760 (1996)).

Resistance to an attack caused by kainic acid (which is an excitatory amino acid receptor agonist) was confirmed in JNK3 knockout mice, and the apoptosis in the hippocampus CA3 region caused by kainic acid, which was found in wild type mice, was not found (*Nature*, 389: 865 (1997)). Based on this, it is considered that the neuronal protection function by the knockout of JNK3 molecule is caused by the disappearance of JNK3 pathway.

Neuronal cell death caused by apoptosis is reported in neurodegenerative diseases. That is, there are reports stating, for example, that neuronal cells undergoing apoptosis are frequently found in the hippocampus of Alzheimer's disease patients (*Experimental Neurology*,

133: 225 (1995)), that fragmentation of DNA and apoptosis-specific changes in nuclei are observed in the brain of Alzheimer's disease patients (*Neuroreport*, 5: 2529 (1994); *Neuroreport*, 6: 1053 (1995)), and that apoptosis of neurons in substantia nigra is observed in Parkinson's disease (*J. Neurol. Sci.*, 137: 120 (1996)).

While JNK3 shows high expression in the brain, JNK1 and JNK2 are expressed in almost all tissues. The c-Jun protein, ATF2 and Elk-1 (one of the transcription factors which control expression of the gene) are considered to be the phosphorylation substrate of JNK1 and JNK2 (*Nature*, 389: 865 (1997)).

Although JNK3 binds to the above phosphorylation substrates, since the binding affinity is weaker than that of JNK1 and JNK2 (*EMBO J.*, 15: 2760 (1996)), it is not clear whether they are true phosphorylation substrates of JNK3 in mammals.

Since the kinase activity of JNK3 can be measured using only the c-Jun protein as substrate and there are few reports in mammals about the binding protein which is considered to interact with JNK3, biochemical reactions using a protein which interacts with JNK3 and controls functions of JNK3 have been hardly studied.

As a polypeptide capable of binding to JNK3 other than the above phosphorylation substrates, JNK/SAPK-associated protein (JSAP1a) has recently been reported



(1997 Annual Meeting of The Molecular Biology Society of Japan (December)).

As described below, it is assumed that JSAP1a functions as a scaffold protein of the JNK3 pathway. As the scaffold protein, JIP-1 (JNK interacting protein-1) which binds to JNK1 and JNK2 is known (*Science*, 281: 1671 (1998)). Variant JIP-1b having a sequence in which 47 amino acids are inserted after the 557th amino acid residue of JIP-1 is also known. The cDNA sequences encoding JIP-1 and JIP-1b have been registered in GenBank data base (accession numbers: AF003115 and AF054611, respectively).

Furthermore, it is known that STE5 protein functions as a scaffold protein in the signal transduction pathway of a mating pheromone of yeast *Saccharomyces cerevisiae*.

That is, STE5 protein is a scaffold protein which binds to all of the kinases STE11 (MAPKKK), STE7 (MAPKK) and FUS/KSS1 (MAPK) that exist in a series of MAP-kinase pathway in which the pheromone transfers a signal after binding to its receptor, thus having a function of transferring the signal efficiently (*Genes Dev.*, 8: 313 (1994); *Cell*, 78: 499 (1994); *Proc. Natl. Acad. Sci., U.S.A.*, 91: 7762 (1994)).

#### DISCLOSURE OF THE INVENTION

An object of the present invention is to provide an agent for preventing or treating neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis and the like, amyotrophic diseases such as amyotrophic lateral sclerosis and the like, ischemic diseases, brain damage due to stroke, schizophrenia, depression, epilepsy, or various immunological and inflammatory diseases, using a novel polypeptide which binds to JNK3 in the JNK3 cascade activated in response to a signal which induces stress and apoptosis, a DNA encoding the polypeptide, an antibody which recognizes the polypeptide and the like.

Activation of the JNK cascade is considered to be one of the mechanisms which cause apoptosis of cells. In addition, in neurodegenerative disease such as Alzheimer's disease or Parkinson's disease, apoptosis can be exemplified as the mechanism of cell death.

Thus, intensive studies were conducted based on a belief that an agent for treating these neurodegenerative diseases and others could be obtained if the JNK3 pathway which is especially highly expressed in the brain could be inhibited and blocked, and that an agent capable of selectively inhibiting and blocking the JNK3 pathway would provide a therapeutic agent having fewer side effects. Accordingly, the present invention has been accomplished.

Specifically, the present invention relates to the following inventions of (1) to (39).

(1) A polypeptide comprising an amino acid sequence selected from the amino acid sequences represented by any one of SEQ ID NOS:10 to 16.

(2) A polypeptide comprising an amino acid sequence in which at least one amino acid has been deleted, substituted or added in an amino acid sequence selected from the amino acid sequences represented by any one of SEQ ID NOS:14 to 16 and is capable of binding to JNK3.

The number of amino acids which have been deleted, substituted or added is not particularly limited in the above; however, they are preferably from 1 to tens, particularly from 1 to several amino acids. Also, in order to bind the polypeptide comprising a deleted, substituted or added amino acid sequence to JNK3 in the same manner as the original polypeptide, it is preferred that it has a homology of at least 60% or more, generally 80% or more, particularly 95% or more, with the amino acid sequence of the original polypeptide. Hereinafter, the amino acid sequence in which at least one amino acid has been deleted, substituted or added means a polypeptide based on the definition described above.

The deletion, substitution or addition of an amino acid can be carried out by the well-known site-specific mutagenesis technique, and specifically, it can be carried

out according to the methods described in *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press (1989) (hereinafter referred to as "*Molecular Cloning*, 2nd ed."), *Current Protocols in Molecular Biology*, Supplement 1-38, John Wiley & Sons (1987-1997) (hereinafter referred to as "*Current Protocols in Molecular Biology*"), *Nucleic Acids Research*, 10: 6487 (1982); *Proc. Natl. Acad. Sci. USA*, 79: 6409 (1982); *Gene*, 34: 315 (1985); *Nucleic Acids Research*, 13: 4431 (1985); *Proc. Natl. Acad. Sci. USA*, 82: 488 (1985); *Proc. Natl. Acad. Sci. USA*, 81: 5662 (1984); *Science*, 224: 1431 (1984); PCT WO 85/00817 (1985); *Nature*, 316: 601 (1985); and the like.

(3) A DNA which encodes the polypeptide of (1) or (2).

(4) A DNA comprising a nucleotide sequence selected from the nucleotide sequences represented by any one of SEQ ID NOS:2 to 8.

(5) A DNA which hybridizes with a DNA comprising the nucleotide sequence represented by any one of SEQ ID NOS:6 to 8 under stringent conditions, and encodes a polypeptide capable of binding to JNK3.

The above "DNA which hybridizes with a DNA comprising the nucleotide sequence represented by any one of SEQ ID NOS:6 to 8 under stringent conditions, and encodes a polypeptide capable of binding to JNK3" means a DNA obtained by colony hybridization, plaque hybridization, Southern blot hybridization or the like using the DNA

described in (3) or (4) as a probe. Examples include DNA which can be identified by carrying out hybridization at 65°C in the presence of 0.7-1.0 M NaCl using a filter on which a DNA prepared from colonies or plaques is immobilized, and then washing the filter with 0.1x to 2x SSC (saline-sodium citrate) solution (the composition of 1x SSC comprises 150 mM sodium chloride and 15 mM sodium citrate) at 65°C.

The hybridization can be carried out according to methods described in experiment texts, such as *Molecular Cloning*, 2nd ed., *Current Protocols in Molecular Biology*, *DNA Cloning 1: Core Techniques, A Practical Approach*, Second Edition, Oxford University Press (1955) and the like.

Specific examples of DNA which can be hybridized include a DNA having a homology of at least 80% or more, preferably 95% or more, with a nucleotide sequence represented by SEQ ID NOS:1 to 8 wherein the homology is calculated using BLAST (*J. Mol Biol.*, 215: 403 (1990)), FAST (*Methods in Enzymology*, 183: 63-69) or the like.

(6) A recombinant DNA obtained by inserting the DNA of any one of (3) to (5) into a vector.

(7) The recombinant DNA according to (6), which is a recombinant DNA selected from plasmid pcDNA3-S-JSAP1b, plasmid pcDNA3-S-JSAP1c, plasmid pcDNA3-S-JSAP4, plasmid pGAD10-JSAP5, and plasmid pcDNA3-His-S-JSAP5.

(8) A transformant comprising the recombinant DNA of (6) or (7).

(9) The transformant according to (8), which is a transformant selected from a microorganism, an animal cell, a plant cell, and an insect cell.

(10) The transformant according to (9), which is a microorganism belonging to the genus *Escherichia*.

(11) The transformant according to (10), wherein the microorganism belonging to the genus *Escherichia* is a microorganism selected from *Escherichia coli* JSAP1b/pcDNA3 (FERM BP-6567), *Escherichia coli* JSAP1c/pcDNA3 (FERM BP-6568), *Escherichia coli* JSAP4/pcDNA3 (FERM BP-6569), *Escherichia coli* JSAP5/pGAD10 (FERM BP-6570), and *Escherichia coli* JSAP5/pcDNA3 (FERM BP-6928).

(12) A method for producing the polypeptide of (1) or (2), comprising culturing the transformant of any one of (8) to (11) in a medium to produce and accumulate the polypeptide of (1) or (2) in the culture, and recovering the polypeptide from the culture.

(13) An oligonucleotide which is selected from an oligonucleotide comprising a sequence identical to continuous 5 to 60 bases in a nucleotide sequence in any one of the DNA's of (3) to (5) and the DNA comprising the nucleotide sequence represented by SEQ ID NO:5, an oligonucleotide comprising a sequence complementary to the

oligonucleotide, and an oligonucleotide analogue of these oligonucleotides.

(14) The oligonucleotide according to (13), wherein the oligonucleotide analogue is selected from oligonucleotide analogues in which: a phosphodiester bond is converted into a phosphorothioate bond, a phosphodiester bond is converted into an N3'-P5' phosphoamidate bond, a ribose-phosphodiester bond is converted into a peptide-nucleic acid bond, uracil is substituted with C-5 propynyluracil, uracil is substituted with C-5 thiazoleuracil, cytosine is substituted with C-5 propynylcytosine, cytosine is substituted with phenoxazine-modified cytosine, ribose is substituted with 2'-O-propylribose, and ribose is substituted with 2'-methoxyethoxyribose.

(15) A method for detecting mRNA encoding the polypeptide of (1) or (2), comprising using the oligonucleotide of (13) or (14).

(16) A method for inhibiting expression of the polypeptide of (1) or (2), comprising using the oligonucleotide of (13) or (14).

(17) An antibody which recognizes the polypeptide of (1) or (2).

(18) A method for immunologically detecting the polypeptide of (1) or (2), comprising using the antibody of (17).

(19) A method for immunohistologically staining of the polypeptide of (1) or (2), comprising using the antibody of (17).

(20) An immunohistologically staining agent, comprising the antibody of (17).

(21) A method of screening a compound having an inhibitory activity on binding of a polypeptide to JNK3, comprising bringing the polypeptide into contact with JNK3 and a test sample, said polypeptide comprising an amino acid sequence selected from the amino acid sequences represented by any one of SEQ ID NOS:9 to 16 or a polypeptide comprising an amino acid sequence in which at least one amino acid has been deleted, substituted or added in an amino acid sequence selected from the amino acid sequences represented by any one of SEQ ID NOS:9 to 16 and being capable of binding to JNK3.

(22) A method of screening a compound having an inhibitory activity on phosphorylation of a polypeptide caused by activated JNK3, comprising bringing the polypeptide into contact with activated JNK3 and a test sample, said polypeptide comprising an amino acid sequence selected from the amino acid sequences represented by any one of SEQ ID NOS:9 to 16 or a polypeptide comprising an amino acid sequence in which at least one amino acid has been deleted, substituted or added in an amino acid sequence selected from the amino acid sequences represented



by any one of SEQ ID NOS:9 to 16 and being capable of binding to JNK3.

(23) A compound obtained by the method of (21) or (22) or a pharmacologically acceptable salt thereof.

According to the description, the pharmacologically acceptable salt of a compound includes pharmacologically acceptable acid addition salts, metal salts, ammonium salts, organic amine addition salts, amino acid addition salts and the like.

(24) A method of screening a compound capable of changing expression of a gene encoding a polypeptide, comprising bringing a cell which expresses the polypeptide into contact with a test sample, said polypeptide comprising an amino acid sequence selected from the amino acid sequences represented by any one of SEQ ID NOS:9 to 16 or a polypeptide comprising an amino acid sequence in which at least one amino acid has been deleted, substituted or added in an amino acid sequence selected from the amino acid sequences represented by SEQ ID NOS:9 to 16 and being capable of binding to JNK3.

(25) The method according to (24), wherein the expression of a gene is detected by the method of (15).

(26) The method according to (24), wherein the polypeptide is detected using the method of (18).

(27) A compound obtained by the method of any one of (24) to (26) or a pharmacologically acceptable salt thereof.

(28) An inhibitor of binding of a polypeptide and JNK3, wherein the polypeptide comprises an amino acid sequence selected from the amino acid sequences represented by any one of SEQ ID NOS:9 to 16 or a polypeptide comprising an amino acid sequence in which at least one amino acid has been deleted, substituted or added in an amino acid sequence selected from the amino acid sequences represented by any one of SEQ ID NOS:9 to 16 and being capable of binding to JNK3.

(29) An inhibitor of phosphorylation of a polypeptide by activated JNK3 a polypeptide, wherein the polypeptide comprises an amino acid sequence selected from the amino acid sequences represented by any one of SEQ ID NOS:9 to 16 or a polypeptide comprising an amino acid sequence in which at least one amino acid has been deleted, substituted or added in an amino acid sequence selected from the amino acid sequences represented by any one of SEQ ID NOS:9 to 16 and being capable of binding to JNK3.

(30) An agent for preventing neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis and the like, amyotrophic diseases, such as amyotrophic lateral sclerosis and the like, ischemic diseases, brain damage due to stroke, schizophrenia, depression, epilepsy, or various immunological and inflammatory diseases, comprising the polypeptide of (1) or (2).

(31) An agent for treating neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis and the like, amyotrophic diseases, such as amyotrophic lateral sclerosis and the like, ischemic diseases, brain damage due to stroke, schizophrenia, depression, epilepsy, or various immunological and inflammatory diseases, comprising the polypeptide of (1) or (2).

(32) An agent for preventing neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis and the like, amyotrophic diseases, such as amyotrophic lateral sclerosis and the like, ischemic diseases, brain damage due to stroke, schizophrenia, depression, epilepsy, or various immunological and inflammatory diseases, comprising the oligonucleotide of (13) or (14).

(33) An agent for treating neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis and the like, amyotrophic diseases, such as amyotrophic lateral sclerosis and the like, ischemic diseases, brain damage due to stroke, schizophrenia, depression, epilepsy, or various immunological and inflammatory diseases, comprising the oligonucleotide of (13) or (14).

(34) An agent for preventing neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease,

Huntington's disease, multiple sclerosis and the like, amyotrophic diseases, such as amyotrophic lateral sclerosis and the like, ischemic diseases, brain damage due to stroke, schizophrenia, depression, epilepsy, or various immunological and inflammatory diseases, comprising the antibody of (17).

(35) An agent for treating neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis and the like, amyotrophic diseases, such as amyotrophic lateral sclerosis and the like, ischemic diseases, brain damage due to stroke, schizophrenia, depression, epilepsy, or various immunological and inflammatory diseases, comprising the antibody of (17).

(36) A promoter DNA which controls transcription of a gene encoding the polypeptide of (1) or (2).

(37) A screening method of a compound capable of changing efficiency of transcription by the promoter DNA of (36), comprising bringing a test sample into contact with a transformant comprising a plasmid containing the promoter DNA and a reporter gene connected to the downstream of the promoter DNA; and measuring a translation product content of the reporter gene.

(38) The method according to (37), wherein the reporter gene is a gene selected from a chloramphenicol

acetyltransferase gene, a  $\beta$ -galactosidase gene, a luciferase gene, and a green fluorescent protein gene.

(39) A compound obtained by the method of (37) or (38) or a pharmacologically acceptable salt thereof.

The present invention is explained in detail below.

<1> Acquisition of the DNA of the present invention and preparation of oligonucleotide

(1) Production of cDNA library

In order to produce a cDNA library, total RNA or mRNA is prepared from a suitable cell or tissue.

As the method for preparing total RNA, a guanidine thiocyanate-cesium trifluoroacetate method (*Methods in Enzymology*, 154: 3 (1987)), an acidic guanidine thiocyanate phenol chloroform (AGPC) method (*Analytical Biochemistry*, 162: 156 (1987); *Experimental Medicine*, 9: 1937 (1991)) and the like can be used.

As the method for preparing mRNA as poly(A)<sup>+</sup> RNA from the total RNA, an oligo(dT)-immobilized cellulose column method (*Molecular Cloning*, 2nd ed.), an oligo-dT latex-aided method and the like can be used.

Also, mRNA can be prepared directly from a tissue or cell by using a kit, such as Fast Track mRNA Isolation Kit (manufactured by Invitrogen), Quick Prep mRNA Purification Kit (manufactured by Pharmacia) or the like.

Examples of suitable cell or tissue include brain cells or brain tissues of animals.

Using the resulting total RNA or mRNA, a cDNA library is produced by a known method.

Examples of the cDNA library production method include methods described in *Molecular Cloning*, 2nd ed., *Current Protocols in Molecular Biology*, *DNA Cloning 1: Core Techniques, A Practical Approach*, 2nd ed., Oxford University Press (1995) and the like, methods using a commercially available kit, such as SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by Gibco BRL) and ZAP-cDNA Synthesis Kit (manufactured by Stratagene) and the like.

Any of phage vectors, plasmid vectors and the like can be used as the cloning vector for producing cDNA library, so long as it can autonomously reproduce in *Escherichia coli* K12.

Specific examples include ZAP Express (manufactured by Stratagene, *Strategies*, 5: 58 (1992)), pBluescript II SK(+) (*Nucleic Acids Research*, 17: 9494 (1989)), Lambda ZAP II (manufactured by Stratagene),  $\lambda$ gt10 and  $\lambda$ gt11 (*DNA Cloning, A Practical Approach*, 1: 49 (1985)),  $\lambda$ TriplEx (manufactured by Clontech),  $\lambda$ ExCell (manufactured by Pharmacia), pT7T318U (manufactured by Pharmacia), pCD2 (*Mol. Cell. Biol.*, 3: 280 (1983)), pUC18 (*Gene*, 33: 103 (1985)), pAMo (*J. Biol. Chem.*, 268: 22782-22787 (1993)), alias

pAMoPRC3Sc (Japanese Published Unexamined Patent Application No. 336963/93), pGAD10 (manufactured by Clontech) and the like.

Any microorganism can be used as the host microorganism, so long as it belongs to *Escherichia coli*. Specific examples include *Escherichia coli* XL1-Blue MRF' (manufactured by Stratagene, *Strategies*, 5: 81 (1992)), *Escherichia coli* C600 (*Genetics*, 39: 440 (1954)), *Escherichia coli* Y1088 (*Science*, 222: 778 (1983)), *Escherichia coli* Y1090 (*Science*, 222: 778 (1983)), *Escherichia coli* NM522 (*J. Mol. Biol.*, 166: 1 (1983)), *Escherichia coli* K802 (*J. Mol. Biol.*, 16: 118 (1966)), *Escherichia coli* JM105 (*Gene*, 38: 275 (1985)), *Escherichia coli* SOLRTM Strain (manufactured by Stratagene), *Escherichia coli* LE392 (*Molecular Cloning*, 2nd ed.) and the like.

In addition to the cDNA library produced by the above method, a commercially available cDNA library can also be used.

Representative cDNA libraries include those derived from organs of human, cattle, mouse, rat, rabbit and the like which are available from Clontech, Life Tech Oriental, Health Science Research Resources Bank, Japan and the like.

(2) Acquisition of the DNA of the present invention

cdNA clone having the DNA of the present invention can be obtained from the cdNA library produced in the above (1) by the following two-hybrid system using yeast.

A full length cdNA encoding JNK3, such as mouse JNK3 (*Nature Medicine*, 3: 89 (1997)), is inserted into a cloning vector containing a sequence encoding the GAL4 DNA binding domain, such as the sequence of pAS2-1 (manufactured by Clontech), and then introduced into yeast CG-1945 (manufactured by Clontech).

By the method of the above (1), a brain-derived cdNA is inserted into a cloning vector containing a sequence encoding the GAL4 transcription activation domain, such as the sequence of pGAD10 (manufactured by Clontech), to produce a cdNA library.

The cdNA library is introduced into the above JNK3-containing CG-1945 to obtain a transformant.

The CG-1945 is a reporter yeast strain having *HIS3* and *lacZ* genes which are under control of the GAL4 response sequence, and the *HIS3* and *lacZ* genes are expressed only when two proteins expressed from hybrid constructs of JNK3 and brain cdNA are bound. Thus, cdNA clone having the DNA of the present invention encoding a polypeptide capable of binding to JNK3 (JSAP: c-Jun N-terminal kinase/stress-activated protein kinase-associated protein) can be selected by selecting a strain from which histidine



requirement has been removed (e.g., which grows on a histidine-free medium) and which has a  $\beta$ -galactosidase activity.

The transformant from which histidine requirement has been removed and which has  $\beta$ -galactosidase activity can be obtained in one step, but the transformant of interest can also be obtained by first selecting a transformant from which histidine requirement has been removed or a transformant which has  $\beta$ -galactosidase activity (primary positive clone), and then selecting a transformant from which histidine requirement has been removed and which has  $\beta$ -galactosidase activity (secondary positive clone).

The brain-derived cDNA fragment of interest is obtained by recovering the pGAD-derived plasmid by a known method from the resulting transformant of interest.

Using the resulting cDNA fragment as the probe and labeling the probe, e.g., with an isotope or fluorescence, the full length cDNA of interest can be obtained by colony hybridization, plaque hybridization (*Molecular Cloning*, 2nd ed.) or the like.

A nucleotide sequence of the DNA obtained by the above method can be determined by inserting the DNA fragment, directly or after digestion with a suitable restriction enzyme or the like, into a vector and analyzing it by a generally used nucleotide sequence analyzing method such as the dideoxy method of Sanger et al. (*Proc. Natl.*

Acad. Sci. USA, 74: 5463 (1997)) or using nucleotide sequence analyzing apparatus manufactured by Perkin Elmer (373A DNA sequencer), LI-COR, Pharmacia or the like.

The DNA obtained by the above method includes DNA comprising the nucleotide sequence represented by any one of SEQ ID NOS:1 to 8.

*Escherichia coli* JSAP1b/pcDNA3 comprising plasmid pcDNA3-S-JSAP1b containing DNA of the nucleotide sequence represented by SEQ ID NO:2, *Escherichia coli* JSAP1c/pcDNA3 comprising plasmid pcDNA3-S-JSAP1c containing DNA of the nucleotide sequence represented by SEQ ID NO:3, *Escherichia coli* JSAP4/pcDNA3 comprising plasmid pcDNA3-S-JSAP4 containing DNA of the nucleotide sequence represented by SEQ ID NO:6 and *Escherichia coli* JSAP5/pGAD10 comprising plasmid pGAD10-JSAP5 containing DNA of the nucleotide sequence represented by SEQ ID NO:7 have been deposited on November 6, 1998, and *Escherichia coli* JSAP5/pcDNA3 comprising plasmid pcDNA3-His-S-JSAP5 containing DNA of the nucleotide sequence represented by SEQ ID NO:8 on November 2, 1999, in National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Higashi 1-1-3, Tsukuba-shi, Ibaraki, Japan (postal code 305-8566), as FERM BP-6567, FERM BP-6568, FERM BP-6569, FERM BP-6570, and FERM BP-6928, respectively.

Also, a DNA of interest derived from other tissue or other animal, such as human, can be obtained by

selecting a DNA which hybridizes under stringent conditions with the DNA obtained by the above method.

The DNA of interest can also be prepared by chemical synthesis using a DNA synthesizer based on the nucleotide sequence information obtained above. Examples of the DNA synthesizer include a DNA synthesizer manufactured by Shimadzu using the thiophosphite method, a DNA synthesizer model 392 manufactured by Perkin Elmer using the phosphoramidite method and the like.

With regard to the novelty of the resulting nucleotide sequence, such can be confirmed by searching a nucleotide sequence data base, such as GenBank, EMBL, DDBJ or the like, using a homology searching program, such as BLAST or the like.

With regard to the novel nucleotide sequences, known genes having the homology can be searched by first converting them into an amino acid sequence and then searching an amino acid sequence data base, such as GenPept, PIR, Swiss-Prot or the like, using a homology searching program, such as FASTA, FrameSearch or the like.

(3) Preparation of the oligonucleotide of the present invention

Oligonucleotides, such as antisense oligonucleotides, sense oligonucleotides and the like, having a partial sequence of the DNA of the present

invention can be prepared by a known method or the above DNA synthesizer using the DNA of the present invention or a DNA fragment thereof obtained above.

Examples of the oligonucleotide include DNA comprising a sequence which is the same as continuous 5 to 60 nucleotides in a nucleotide sequence of the above DNA, and DNA comprising a sequence complementary to the DNA. Specific examples include DNA comprising a continuous 5 to 60 nucleotides in a nucleotide sequence represented by SEQ ID NOS:1 to 8, and DNA having a sequence complementary to such DNA.

When the oligonucleotide is used as the sense primer and antisense primer, the above oligonucleotide in which melting point ( $T_m$ ) and the number of nucleotides are not significantly different from each other is preferred.

Furthermore, analogues of the oligonucleotide can also be used as the oligonucleotide of the present invention.

Examples of the oligonucleotide analogues include oligonucleotide analogues in which: a phosphodiester bond is converted to a phosphorothioate bond, a phosphodiester bond is converted to an N3'-P5' phosphoamidate bond, ribose and a phosphodiester bond is converted to a peptide nucleic acid bond, uracil is substituted with C-propynyluracil, uracil is substituted with C-5 thiazoluracil, cytosine is substituted with C-5 propynylcytosine, cytosine is

substituted with phenoxazin-modified cytosine, ribose is substituted with 2'-O-propylribose, ribose is substituted with 2'-methoxyethoxyribose and the like (*Cell Engineering*, 16: 1463 (1997)).

<2> Preparation of the polypeptide of the present invention

(1) Production of transformant

Methods described in *Molecular Cloning*, 2nd ed., *Current Protocols in Molecular Biology* and the like can be used to express the DNA of the present invention encoding JSAP obtained by the method described in <1> above and produce the polypeptide of the present invention.

Specifically, a recombinant vector to which the DNA of the present invention has been inserted into downstream of the promoter of a suitable expression vector is constructed, a transformant expressing the polypeptide of the present invention is obtained by introducing the vector into a host cell, and the transformant is cultured to produce the polypeptide of the present invention.

Any of bacteria, yeast, animal cells, insect cells, plant cells and the like can be used as the host cell, so long as it can express the gene of interest.

Examples of the expression vector include those which can autonomously replicate in the above host cell or which can be integrated into a chromosome and have a

promoter at an operative position such that the DNA of the present invention can be transcribed.

When prokaryote, such as a bacterium or the like, is used as the host cell, it is preferred that the expression vector of the polypeptide gene of the present invention can autonomously replicate in the prokaryote and is a recombinant vector constructed with a promoter, a ribosome binding sequence, the DNA of the present invention and a transcription termination sequence. A promoter-controlling gene can also be utilized.

Examples of the expression vector include pBTrp2, pBTac1 and pBTac2 (all available from Boehringer Mannheim), pKK233-2 (manufactured by Pharmacia), pSE280 (manufactured by Invitrogen), pGEMEX-1 (manufactured by Promega), pQE-8 (manufactured by QIAGEN), pKYP10 (Japanese Published Unexamined Patent Application No. 110600/83), pKYP200 (*Agric. Biol. Chem.*, 48: 669 (1984)), pLSA1 (*Agric. Biol. Chem.*, 53: 277 (1989)), pGEL1 (*Proc. Natl. Acad. Sci. USA*, 82: 4306 (1985)), pBluescript II SK(-) (manufactured by Stratagene), pTrs32 (FERM BP-5408), pGHA2 (FERM B-400), pGKA2 (FERM BP-6798), pTerm2 (Japanese Published Unexamined Patent Application No. 22979/91, US 4686191, US 4939094, US 5160735), pGEX (manufactured by Pharmacia), pET (manufactured by Novagen), pSupex, pUB110, pTP5, pC194, pTrxFus (manufactured by Invitrogen), pMAL-c2 (manufactured by New England Biolabs) and the like.

With regard to the promoter, any promoter can be used, so long as it can express in a host cell, such as *Escherichia coli*, *Bacillus subtilis* and the like. Examples include promoters derived from *Escherichia coli*, phage and the like, such as *trp* promoter (*P<sub>trp</sub>*), *lac* promoter (*P<sub>lac</sub>*), *P<sub>L</sub>* promoter, *P<sub>R</sub>* promoter, T7 promoter and the like; SP01 promoter; SP02 promoter; *penP* promoter; and the like. Also, artificially designed and modified promoters, such as a promoter in which two *P<sub>trp</sub>* are linked in series (*P<sub>trp</sub>x2*), *tac* promoter, *lacT7* promoter, *letI* promoter or the like, can be used.

As the ribosome binding sequence, it is preferred to use a plasmid in which the space between Shine-Dalgarno sequence and the initiation codon is adjusted to a suitable distance (for example, 6 to 18 nucleotides).

The transcription termination sequence is not required for the expression of the DNA of the present invention. However, the transcription terminating sequence is preferably arranged at just downstream of the structural gene.

Examples of the host cell include microorganisms belonging to the genus *Escherichia*, the genus *Serratia*, the genus *Bacillus*, the genus *Brevibacterium*, the genus *Corynebacterium*, the genus *Microbacterium*, the genus *Pseudomonas* and the like. Specific examples include *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue,

*Escherichia coli* DH1, *Escherichia coli* MC1000, *Escherichia coli* KY3276, *Escherichia coli* W1485, *Escherichia coli* JM109, *Escherichia coli* HB101, *Escherichia coli* No.49, *Serratia ficaria*, *Serratia fonticola*, *Serratia liquefaciens*, *Serratia marcescens*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Brevibacterium ammoniagenes*, *Brevibacterium immariophilum* ATCC 14068, *Brevibacterium saccharolyticum* ATCC 14066, *Corynebacterium glutamicum* ATCC 13032, *Corynebacterium glutamicum* ATCC 14067, *Corynebacterium glutamicum* ATCC 13869, *Corynebacterium acetoacidophilum* ATCC 13870, *Microbacterium ammoniaphilum* ATCC 15354, *Pseudomonas* sp. D-0110 and the like.

Any method can be used in the method for introducing the recombinant vector, so long as it is a method for introducing DNA into the above host cell. Examples include a method using a calcium ion (*Proc. Natl. Acad. Sci. USA*, 69: 2110 (1972)), a protoplast method (Japanese Published Unexamined Patent Application No. 248394/88), an electroporation method (*Gene*, 17: 107 (1982); and *Molecular & General Genetics*, 168: 111 (1979)) and the like.

When yeast is used as the host cell, examples of expression vector include YEp13 (ATCC 37115), YEp24 (ATCC 37051), YCp50 (ATCC 37419), pHS19, pHS15 and the like.

Any promoter can be used so long as it can express in yeast. Examples include PH05 promoter, PGK promoter,



GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, a heat shock polypeptide promoter, MFa1 promoter, CUP 1 promoter and the like.

Examples of the host cell include yeast strains belonging to the genus *Saccharomyces*, the genus *Schizosaccharomyces*, the genus *Kluyveromyces*, the genus *Trichosporon*, the genus *Schwanniomyces* and the like. Specific examples include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Trichosporon pullulans*, *Schwanniomyces alluvius*, *Pichia pastoris* and the like.

Any method can be used as the method for introducing the recombinant vector, so long as it is a method for introducing DNA into yeast. Examples include an electroporation method (*Methods in Enzymology*, 194: 182 (1990)), a spheroplast method (*Proc. Natl. Acad. Sci. USA*, 81: 4889 (1978)), a lithium acetate method (*Journal of Bacteriology*, 153: 163 (1983)) and the like.

When an animal cell is used as the host, examples of expression vector include pcDNAI/Amp (manufactured by Invitrogen), pcDNAI and pcDM8 (*Nature*, 329: 840 (1987)), pAGE107 (Japanese Published Unexamined Patent Application No. 22979/91; *Cytotechnology*, 3: 133 (1990)), pREP4 (manufactured by Invitrogen), pAGE103 (*Journal of Biochemistry*, 101: 1307 (1987)), pAMo, pAMoA, pAS3-3

(Japanese Published Unexamined Patent Application No. 227075/90) and the like.

Any promoter can be used as the method for introducing the recombinant vector, so long as it can express in the animal cell. Examples include a promoter of IE (immediate early) gene of cytomegalovirus (CMV), an early promoter of SV40, a metallothionein promoter, a promoter of retrovirus, a heat shock promoter, SR $\alpha$  promoter and the like. Also, the enhancer of the IE gene of human CMV can be used together with the promoter.

Examples of the animal cell include mouse myeloma cell, rat myeloma cell, mouse hybridoma cell, human Namalwa cell, human Namalwa KJM-1 cell, human fetal kidney cell, human leukemic cell, African grivet kidney cell, Chinese hamster CHO cell, HBT5637 (Japanese Published Unexamined Patent Application No. 299/88) and the like.

Examples of the mouse myeloma cell include SP2/0, NS0 and the like. Examples of the rat myeloma cell include YB2/0 and the like. Examples of the human fetal kidney cell include BALL-1 and the like. Examples of African grivet kidney cell include COS-1, COS-7 and the like.

Any method can be used as the method for introducing the recombinant vector into an animal cell, so long as it is a method for introducing DNA into an animal cell. Examples include an electroporation method (Cytotechnology, 3: 133 (1990)), a calcium phosphate method

(Japanese Published Unexamined Patent Application No. 227075/90), a lipofection method (*Proc. Natl. Acad. Sci. USA*, 84: 7413 (1987)), the method described in *Virology*, 52: 456 (1973), and the like.

When an insect cell is used as the host, the polypeptide can be expressed by a known method described in, for example, *Baculovirus Expression Vectors, A Laboratory Manual*, W.H. Freeman and Company, New York (1992), *Molecular Biology, A Laboratory Manual, Current Protocols in Molecular Biology, Bio/Technology*, 6: 47 (1988) or the like.

Specifically, a recombinant gene transfer vector and baculovirus are co-transfected into an insect cell to obtain a recombinant virus in an insect cell culture supernatant, and then the insect cell is infected with the resulting recombinant virus to express of the polypeptide.

Examples of the gene transfer vector used in the method include pVL1392, pVL1393, pBlueBacIII (all manufactured by Invitrogen) and the like.

Examples of the baculovirus include *Autographa californica* nuclear polyhedrosis virus which infects insects of the family *Barathra* and the like.

Examples of the insect cell include *Spodoptera frugiperda* ovary cell, *Trichoplusia ni* ovary cell, *Bombyx mori* ovary-derived culturing cell and the like.

Examples of *Spodoptera frugiperda* ovary cell include Sf9 and Sf21 (*Baculovirus Expression Vectors, A Laboratory Manual*) and the like. Examples of *Trichoplusia ni* ovary cell include High 5 and BTI-TN-5B1-4 (manufactured by Invitrogen) and the like. Examples of the *Bombyx mori* ovary-derived culturing cell include *Bombyx mori* N4 and the like.

The method for co-transfecting the above recombinant gene transfer vector and the above baculovirus for the preparation of the recombinant virus include a calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), a lipofection method (*Proc. Natl. Acad. Sci. USA*, 84: 7413 (1987)) and the like.

As the gene expression method, a secreted protein production, a fusion protein expression and the like can be effected according to the method described in *Molecular Cloning*, 2nd ed., in addition to direct expression.

When the expression is carried out in yeast, an animal cell or an insect cell, a glycosylated polypeptide can be obtained.

The resulting transformant is cultured in a medium, the polypeptide of the present invention is formed and accumulated in the medium, and the resulting polypeptide is recovered to produce the polypeptide of the present invention.

Furthermore, a suitable expression vector for expressing the polypeptide of the present invention can be introduced into a cell obtained from the body of a patient, and then the cell is returned into the patient to express the polypeptide of the present invention in the patient's living body.

(2) Culturing of the transformant

Culturing the transformant of the present invention in a medium is carried out according to a known method used in culturing of the host.

As a medium for culturing the transformant obtained by using, as the host, prokaryote (such as *Escherichia coli* or the like) or eukaryote (such as yeast or the like), the medium may be either a natural medium or a synthetic medium, so long as it contains a carbon source, a nitrogen source, an inorganic salt and the like which can be assimilated by the organism and the transformant can be cultured efficiently.

Any source can be used as the carbon source, so long as it can be assimilated by the organism. Examples include carbohydrates, such as glucose, fructose, sucrose, molasses containing them, starch, starch hydrolysate and the like; organic acids, such as acetic acid, propionic acid and the like; alcohols, such as ethanol, propanol and the like; and so on.

Examples of the nitrogen source include ammonia; ammonium salts of inorganic acids or organic acids, such as ammonium chloride, ammonium sulfate, ammonium acetate, ammonium phosphate and the like, other nitrogen-containing compounds, peptone, meat extract, yeast extract, corn steep liquor, casein hydrolysate, soybean meal and soybean meal hydrolysate, various fermented cells and hydrolysates thereof and the like.

Examples of the inorganic salt include potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, calcium carbonate and the like.

Culturing is generally carried out under aerobic conditions by shaking culture, aeration stirring culture or the like. The culturing temperature is preferably from 15 to 40°C, and the culturing time is generally from 16 to 96 hours. The pH is maintained at 3.0 to 9.0 during the culturing. The pH is adjusted using inorganic or organic acid, an alkali solution, urea, calcium carbonate, ammonia or the like.

Also, antibiotics, such as ampicillin, tetracycline and the like, can be added to the medium during the culturing, if desired.

When a microorganism transformed with an expression vector using an inducible promoter as the promoter is

cultured, an inducer can be added to the medium, if desired. For example, when a microorganism transformed with an expression vector using *lac* promoter is cultured, isopropyl- $\beta$ -D-thiogalactopyranoside or the like can be added to the medium, and when a microorganism transformed with an expression vector using *trp* promoter is cultured, indoleacrylic acid or the like can be added to the medium.

Examples of the medium for culturing a transformant obtained using an animal cell as the host include generally used RPMI 1640 medium (*The Journal of the American Medical Association*, 199: 519 (1967)), Eagle's MEM (*Science*, 122: 501 (1952)), DMEM (*Virology*, 8: 396 (1959)), and 199 Medium (*Proceeding of the Society for the Biological Medicine*, 73: 1 (1950)), media to which fetal calf serum or the like has been added to these media and the like.

Culturing is generally carried out under conditions at pH of 6 to 8 and at 30 to 40°C for 1 to 7 days in the presence of 5% CO<sub>2</sub> or the like.

Furthermore, if desired, antibiotics, such as kanamycin, penicillin, streptomycin and the like, can be added to the medium during the culturing.

Examples of the medium for culturing a transformant obtained using an insect cell as the host include generally used TNM-FH medium (manufactured by Pharmingen), Sf-900 II SFM (manufactured by Life Technologies), ExCell 400 and

ExCell 405 (both manufactured by JRH Biosciences), Grace's Insect Medium (*Nature*, 195: 788 (1962)) and the like.

Culturing is generally carried out under conditions at pH of 6 to 7 and at 25 to 30°C for 1 to 5 days or the like.

Furthermore, if desired, antibiotics, such as gentamicin and the like, can be added to the medium during the culturing.

### (3) Isolation and purification of the expressed polypeptide

When the polypeptide of the present invention expressed by the above method is isolated and purified from the above culture medium of the transformant, usual methods for isolating and purifying enzymes can be used.

For example, when the polypeptide of the present invention is expressed in a dissolved state inside the cells, the cells after completion of the culturing are recovered by centrifugation, suspended in an aqueous buffer and then disrupted using an ultrasonic oscillator, a French press, a Manton Gaulin homogenizer, a dynamill or the like to obtain a cell-free extract.

A purified product can be obtained from a supernatant prepared by centrifuging the cell-free extract with a technique, such as solvent extraction; salting out with ammonium sulfate or the like; desalting; precipitation with an organic solvent or the like; anion exchange



chromatography using a resin, such as diethylaminoethyl (DEAE)-Sephacrose, DIAION HPA-75 (manufactured by Mitsubishi Chemical), etc.; cation exchange chromatography using a resin, such as S-Sephacrose FF (manufactured by Pharmacia), etc.; hydrophobic chromatography using a resin, such as butyl-Sephacrose, phenyl-Sephacrose, etc.; gel filtration using a molecular sieve; affinity chromatography; chromatofocusing; electrophoresis, such as isoelectric focusing, etc.; or the like, alone or in combination.

Also, when the polypeptide is expressed inside the cells in the form of an inclusion body, the cells are recovered and then disrupted in the same manner, the polypeptide is recovered from the precipitate fraction obtained by centrifuging the cells, and then the inclusion body of the polypeptide is solubilized with a protein modifier.

The solubilized solution is diluted or dialyzed to a solution containing no protein denaturant or a solution containing such a low concentration of the polypeptide denaturant that the protein is not denatured, the polypeptide having a normal stereostructure is folded, and then a purified product is obtained by an isolation and purification method similar to the above.

When the polypeptide of the present invention or an analogue thereof, such as a glycosylated product or the like, is secreted extracellularly, the polypeptide of the

present invention or the analogue thereof, such as the glycosylated product or the like, can be recovered from the culture supernatant.

That is, the soluble fraction is obtained by treating the culture by a technique, such as centrifugation or the like, in the same manner as the above, and a purified product can be obtained from the soluble fraction by an isolation and purification method similar to the above.

Furthermore, a fusion protein of the polypeptide of the present invention and other protein is produced, and it can be purified using affinity chromatography using a substance having affinity to the fusion protein. For example, a fusion protein of the present invention and protein A is produced according to the method of Lowe *et al.* (*Proc. Natl. Acad. Sci. USA*, 86: 8227 (1989); *Genes Develop.*, 4: 1288 (1990)), or the method described in Japanese Published Unexamined Patent Application No. 336963/93 or 823021/94, and it can be purified by affinity chromatography using immunoglobulin G.

Moreover, the polypeptide of the present invention is produced as a fusion protein with Flag peptide, and the fusion protein can be purified by affinity chromatography using an anti-Flag antibody. Further purification can be carried out by affinity chromatography using the antibody against the polypeptide *per se*.

Furthermore, the polypeptide of the present invention can be produced by a chemical synthesis method, such as Fmoc method (fluorenylmethyloxycarbonyl method), tBoc method (t-butyloxycarbonyl method) or the like.

Also, the polypeptide can be chemically synthesized using a peptide synthesizer manufactured by Advanced ChemTech, Perkin-Elmer, Pharmacia, Protein Technology Instrument, Synthecell-Vega, Shimadzu Corporation or the like.

The structure of the purified polypeptide of the present invention is determined by any method generally used in the field of protein chemistry, for example, the method described in *Protein Structure Analysis for Gene Cloning* (Hisashi Hirano, Tokyo Kagaku Dojin, 1993).

<3> Preparation of antibody which recognizes the polypeptide of the present invention

(1) Preparation of polyclonal antibody

A purified product of a full length or a partial fragment of the polypeptide obtained by the method described in the above <2> is used as an antigen, and administered to an animal to produce a polyclonal antibody.

The peptide used as the antigen can be produced by a peptide synthesizer based on the amino acid sequence of the polypeptide of the present invention.

A purified product of a full length or a partial fragment of the polypeptide obtained by phosphorylating the polypeptide obtained in the method described in the above <2> using activated JNK3 described below is used as an antigen, and administered to an animal to produce a polyclonal antibody which specifically recognizes the phosphorylated polypeptide of the present invention.

Examples of the phosphorylated polypeptide of the present invention include the amino acid sequence represented by SEQ ID NO:9 in which at least one of 234th, 244th and 255th amino acids has been phosphorylated, the amino acid sequence represented by SEQ ID NO:10 in which at least one of 243rd, 253rd and 264th amino acids has been phosphorylated, the amino acid sequence represented by SEQ ID NO:11 in which at least one of 266th, 276th and 287th amino acids has been phosphorylated, and the amino acid sequence represented by SEQ ID NO:12 in which at least one of 265th, 275th and 286th amino acids has been phosphorylated.

Examples of the animal to be administered include rabbits, goats, 3- to 20-week old rats, mice, hamsters and the like.

The dose of the antigen is preferably 50 to 100  $\mu$ g per animal.

When the peptide is used, the peptide which covalently binds to a carrier protein, such as keyhole

limpet haemocyanin, bovine thyroglobulin or the like, is preferably used as the antigen.

The administration of the antigen is carried out 3 to 10 times at the intervals of 1 or 2 weeks after the first administration. Three to seven days after each administration, blood is collected from the venous plexus of the eyeground, and it is confirmed that the serum reacts with the antigen by the enzyme immunoassay (*Enzyme Immunoassay (ELISA)*, Igaku Shoin (1988), *Antibodies - A Laboratory Manual*, Cold Spring Harbor Laboratory (1988)) or the like.

A serum is obtained from a non-human mammal having a sufficient antibody titer against the antigen used for the immunization, and the serum is isolated and purified to obtain a polyclonal antibody.

The methods for the isolation and purification include centrifugation, salting out by 40-50% ammonium sulfate, caprylic acid precipitation (*Antibodies, A Laboratory manual*, Cold Spring Harbor Laboratory (1988)), and chromatography using a DEAE-Sepharose column, an anion exchange column, a protein A or G-column, a gel filtration column and the like, which are used alone or in combination.

## (2) Preparation of monoclonal antibody

### (2-1) Preparation of antibody-producing cell

Three to seven days after the antigen substance is finally administered to a rat having a serum showing a sufficient antibody titer, the rat spleen is excised.

The spleen is cut to pieces in MEM (manufactured by Nissui Pharmaceutical), loosened using a pair of forceps, followed by centrifugation at 1,200 rpm for 5 minutes, and the resulting supernatant is discarded.

The spleen cells in the precipitated fraction is treated with a Tris-ammonium chloride buffer (pH 7.65) for 1 to 2 minutes to eliminate erythrocytes and washed three times with MEM, and the resulting spleen cells are used as antibody-producing cells.

### (2-2) Preparation of myeloma cells

An established cell line derived from a mouse or a rat is used as myeloma cells. Examples include 8-azaguanine-resistant mouse (BALB/c) myeloma cell lines, such as P3-X63Ag8-U1 (P3-U1) (*Curr. Topics in Microbiol. Immunol.*, 81: 1 (1978), *Eur. J. Immunol.*, 6: 511 (1976)), SP2/O-Ag14 (SP-2) (*Nature*, 276: 269 (1978)), P3-X63-Ag8653 (653) (*J. Immunol.*, 123: 1548 (1979)), P3-X63-Ag8 (X63) (*Nature*, 256: 495 (1975)) and the like. These cell lines were subcultured in a 8-azaguanine medium (medium in which, to a medium obtained by adding glutamine (1.5 mM), 2-

mercaptoethanol ( $5 \times 10^{-5}$  M), gentamicin (10  $\mu$ g/ml) and fetal calf serum (FCS) (manufactured by CSL, 10%) to RPMI-1640 medium (hereinafter referred to as the "normal medium"), 8-azaguanine (15  $\mu$ g/ml) has been further added) and cultured in the normal medium 3 or 4 days before cell fusion, and  $2 \times 10^7$  or more of the cells are used for the fusion.

### (2-3) Production of hybridoma

The antibody-producing cells obtained in (2-1) and the myeloma cells obtained in (2-2) are washed with MEM or PBS (disodium hydrogen phosphate: 1.83 g, sodium dihydrogen phosphate: 0.21 g, sodium chloride: 7.65 g, distilled water: 1 liter, pH: 7.2) and mixed to give a ratio of antibody-producing cells : myeloma cells = 5 to 10 : 1, followed by centrifugation at 1,200 rpm for 5 minutes, and the supernatant is discarded.

The cells in the resulting precipitated fraction were thoroughly loosened, 0.2 to 1 ml of a mix solution of 2 g polyethylene glycol-1000 (PEG-1000), 2 ml MEM and 0.7 ml dimethylsulfoxide (DMSO) per  $10^8$  antibody-producing cells is added to the cells under stirring at 37°C, and then 1 to 2 ml of MEM is further added thereto several times at 1 to 2 minute intervals.

After the addition, MEM is added to prepare a total amount of 50 ml.

The resulting prepared solution is centrifuged at 900 rpm for 5 minutes, and then the supernatant is discarded.

The cells in the resulting precipitated fraction were gently loosened and then gently suspended in 100 ml of HAT medium (the normal medium to which hypoxanthine ( $10^{-4}$  M), thymidine ( $1.5 \times 10^{-5}$  M) and aminopterin ( $4 \times 10^{-7}$  M) have been added) by drawing up into and discharging from a measuring pipette.

The suspension is poured into a 96 well culture plate at 100  $\mu$ l/well and cultured at 37°C for 7 to 14 days in a 5% CO<sub>2</sub> incubator.

After culturing, a part of the culture supernatant is recovered, and a hybridoma which specifically reacts with an antigen used for the immunization is selected to obtain the above antibody-producing cells according to the enzyme immunoassay described in *Antibodies, A Laboratory manual*, Cold Spring Harbor Laboratory Press, Chapter 14 (1998) or the like.

A specific example of the enzyme immunoassay is described below.

A full length or a partial fragment of the polypeptide of the present invention used as the antigen in the immunization is coated on a suitable plate, the hybridoma-culturing supernatant or a purified antibody described in (2-4) below is allowed to react therewith as



the first antibody, an anti-rat immunoglobulin antibody labeled with biotin, an enzyme, a chemiluminescent substance, a radioactive compound or the like is allowed to react therewith as the second antibody, and a hybridoma which specifically reacts with the polypeptide of the present invention is selected as a hybridoma capable of producing the monoclonal antibody against the polypeptide of the present invention.

Cloning is repeated using the hybridoma twice by limiting dilution analysis first in HT medium (a medium in which aminopterin has been removed from HAT medium) and then using normal medium, and a hybridoma which has a stable and sufficient antibody titer is selected as a hybridoma capable of producing the monoclonal antibody of the present invention.

#### (2-4) Preparation of monoclonal antibody

The hybridoma cells capable of producing the polypeptide of the present invention obtained in (2-3) are injected intraperitoneally into a 8- to 10-week-old mouse or a nude mouse intraperitoneally treated with 0.5 ml of 2,6,10,14-tetramethylpentadecane (pristane), followed by 2 weeks of feeding at  $5 \times 10^6$  to  $20 \times 10^6$  cells/animal. The hybridoma causes ascites tumor in 10 to 21 days.

The ascitic fluid is collected from the mouse or nude mouse, and centrifuged to remove solid contents.

A monoclonal antibody can be purified and isolated from the resulting supernatant according to the method similar to that used in the polyclonal antibody.

The subclass of the antibody can be determined using a mouse monoclonal antibody typing kit or a rat monoclonal antibody typing kit. The protein amount can be determined by the Lowry method or by calculation based on the absorbance at 280 nm.

<4> Screening method of useful medicament using novel polypeptide which can bind to JNK3

(1) Preparation of fusion polypeptide between tag polypeptide and the polypeptide of the present invention used for screening

1) Preparation of fusion polypeptide with thioredoxin•S-tag (hereinafter referred to as "Trx•S") peptide

A vector capable of expressing a Trx•S-JSAP fusion polypeptide can be prepared by inserting a full length or a partial length DNA of cDNA encoding the polypeptide capable of binding to JNK3 (JSAP) obtained in <1> above into the downstream of the Trx•S sequence of an expression vector containing the Trx•S sequence, such as pET32 (manufactured by Novagen). In the same manner, a vector capable of expressing a Trx•S-ATF2-JSAP fusion polypeptide can be prepared by inserting the same into the downstream of a Trx•S-ATF2 sequence of an expression vector containing the

Trx•S-ATF2 sequence, such as pET32a (manufactured by Novagen).

The fusion polypeptide can be obtained using the resulting expression vector according to the method described in <2> above.

## 2) Preparation of fusion polypeptide with S-tag peptide

A vector capable of expressing an S-JSAP fusion polypeptide can be prepared by inserting a full length or a partial length DNA of cDNA encoding JSAP into the downstream of the S-tag sequence of an expression vector containing the S-tag sequence, such as an s-modified pcDNA3 in which the S-tag sequence has been inserted into pcDNA3 (manufactured by Invitrogen).

The fusion polypeptide can be obtained using the resulting expression vector according to the method described in <2> above.

## 3) Preparation of fusion polypeptide with GAL4AD peptide

A vector capable of expressing a GAL4AD-JSAP fusion polypeptide can be prepared by inserting a full length or a partial length DNA of cDNA encoding JSAP into the downstream of a GAL4AD sequence of an expression vector containing the GAL4AD sequence, such as pGAD10 (manufactured by Clontech).

The fusion polypeptide can be obtained using the resulting expression vector according to the method described in <2> above.

#### 4) Preparation of fusion polypeptide with Flag peptide

A vector capable of expressing a Flag-JSAP fusion polypeptide can be prepared by inserting a full length or a partial length DNA of cDNA encoding JSAP into the downstream of a Flag sequence of an expression vector containing the Flag sequence, such as pFlag-CMV-2 (manufactured by Kodak) or Flag-modified pcDNA3 vector (manufactured by Invitrogen) in which a Flag tag sequence has been inserted into pcDNA3.

The fusion polypeptide can be obtained using the resulting expression vector according to the method described in <2> above.

#### 5) Preparation of fusion polypeptide with glutathione S-transferase (hereinafter referred to as "GST")

A vector capable of expressing a GST-JSAP fusion polypeptide can be prepared by inserting a full length or a partial length DNA of cDNA encoding JSAP into the downstream of a GST sequence of an expression vector containing the GST sequence, such as pGEX or pGEX-3X (both manufactured by Pharmacia).

The fusion polypeptide can be obtained using the resulting expression vector according to the method described in <2> above. The fusion polypeptide can be purified using a Glutathione Sepharose 6B column (manufactured by Pharmacia).

6) Preparation of fusion polypeptide with Myc tag peptide

A vector capable of expressing a Myc-JSAP fusion polypeptide can be prepared by inserting a full length or a partial length DNA of cDNA encoding JSAP into the downstream of a Myc tag sequence of an expression vector containing the Myc tag sequence, such as a Myc-modified pCDNA3. Myc-modified pCDNA3 (manufactured by Invitrogen) is pCDNA3 modified by connecting a Myc tag code sequence to the upstream of the gene to express the protein to which the tag has been added.

The fusion polypeptide can be obtained using the resulting expression vector according to the method described in <2> above.

7) Preparation of fusion polypeptide with His-S-tag peptide

A vector capable of expressing a His-S-JSAP fusion polypeptide can be prepared by inserting a full length or a partial length DNA of cDNA encoding JSAP into the downstream of a His-S-tag sequence of an expression vector containing the His-S-tag sequence, such as a His-S-modified

pcDNA3. His-S-modified pcDNA3 (manufactured by Invitrogen) is pcDNA3 modified by connecting a His-S tag code sequence to the upstream of the gene to express the protein to which the tag has been added.

The fusion polypeptide can be obtained using the resulting expression vector according to the method described in <2> above.

According to the methods of the above 1) to 7), fusion polypeptides with the polypeptides related to the MAP kinase cascade can be prepared in the same manner.

## (2) Preparation of activated JNK3

A vector is prepared by inserting a DNA encoding the full length or a partial length of Flag-JNK3 prepared according to the method of <4>(1) above into pFlag-CMV-2.

Also, a DNA encoding a  $\Delta$ MEKK1 (a peptide containing from the 1169th to 1488th amino acid residues of MEKK1 and constantly activated) is inserted into expression vector pEF-BOS.

COS-7 cells are transfected with both of the resulting expression vectors using TransIT-LT1 (manufactured by Mirus), and the resulting COS-7 cells are cultured according to a known method to temporally express the Flag-JNK3 fusion polypeptide and  $\Delta$ MEKK1.

After culturing for 24 to 48 hours, the cells are dissolved in a buffer B (50 mM HEPES (pH 7.5), 150 mM NaCl,

1% NP-40, 10% glycerol, 2 mM  $\text{MgCl}_2$ , 1 mM EGTA, 20 mM  $\beta$ -glycerophosphate, 2 mM  $\text{Na}_3\text{VO}_4$ , 1 mM PMSF, 0.2 mM DTT), and the activated Flag-JNK3 is purified using an anti-Flag antibody column (manufactured by Kodak).

The activated Flag-JNK3 can be stored in a buffer containing from 20 to 50% glycerin or a buffer containing no glycerin at  $-20^\circ\text{C}$  to  $-80^\circ\text{C}$ , and can be used by thawing prior to use.

### (3) Screening system using JNK3 binding activity as marker

Since the JNK3 pathway can be inhibited by finding a compound which inhibits binding of JNK3 with each of JSAP obtained in <2> above, it is useful in preventing and treating a disease caused by the JNK3 pathway such as apoptosis or the like.

The method of screening a compound having an activity of inhibiting each of JSAP with JNK3 is explained in detail below.

#### 1) Screening system 1 (method using cultured cells)

Transfection of COS-7 cells with the S-JSAP expression vector obtained according to the method of <4>(1) above and the Flag-JNK3 expression vector is carried out using TransIT-LT1 (manufactured by Mirus), and the resulting COS-7 cells are cultured according to a known method in the presence or absence of a test compound to

transiently express the S-JSAP and Flag-JNK3 fusion polypeptide.

After culturing for 24 to 48 hours, the cultured cells are dissolved in the buffer B, S-protein agarose is added thereto, and then S-JSAP and polypeptide which binds to S-JSAP are precipitated and recovered.

The recovered polypeptide fraction is separated by SDS-PAGE and transferred on membrane Immobilon-P (manufactured by Millipore).

Western blotting is carried out using the membrane and anti-Flag M5 monoclonal antibody (manufactured by Kodak) as the probe, and the polypeptide which binds to the antibody (Flag-JNK3) is visualized using an ECL detection system (manufactured by Amersham) to determine the amount of JNK3 bound to JSAP.

Accordingly, a compound which inhibits binding with JNK3 can be detected using as a control the case in which the test compound is not added.

Examples of the test sample include synthetic compounds, natural proteins, artificially synthesized proteins, peptides, saccharides, lipids and modified products and derivatives thereof, as well as urine, body fluids, tissue extracts, cell culture supernatants and cell extracts of mammals (e.g., mouse, rat, guinea pig, hamster, swine, sheep, bovine, horse, dog, cat, monkey, human and the like), and also non-peptide compounds, fermentation



products, extracts of plants and other organisms and the like. In the screening method described below, the test sample similar to these can be used.

## 2) Screening system 2 (ELISA)

The GST-JSAP fusion polypeptide prepared according to the method in <4>(1) above as such, or a JSAP polypeptide fragment obtained by digesting it with a protease factor Xa (manufactured by Sigma), is used in the binding reaction. Hereinafter, these polypeptides are called JSAP-related polypeptides.

The polypeptides can be stored at -80°C to -20°C in a buffer containing from 20 to 50% of glycerin or a buffer containing no glycerin, which can be used by thawing prior to use.

Each of the above JSAP-related polypeptides is added to a 96 well plate.

The activated JNK3 prepared in <4>(2) is (or the activated JNK3 and a test compound are) added to a buffer such as a binding buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40), followed by stirring.

The thus stirred solution is added to the above plate.

After the addition, the plate is incubated at 4°C for 1 to 2 hours.

After incubation, the plate is washed three times with the same buffer, and the remaining activated JNK3 is determined by ELISA.

For example, the amount of the remaining activated JNK3 can be determined by ELISA using Phospho-SAPK/JNK(Thr183/Tyr185) antibody (manufactured by New England Biolabs) capable of recognizing the activated JNK3 as the first antibody, and using an antibody capable of recognizing the above antibody as the second antibody.

By the method, a compound which inhibits binding with JNK3 can be detected using as a control the case in which the test compound is not added.

#### (4) Screening system using phosphorylation of JSAP1 by JNK3 as marker

Because of the information regarding a) to c) obtained by Examples described below, establishment of a method for screening a compound having an inhibitory activity on phosphorylation of JSAP1 is important in detecting a compound useful in preventing and treating a disease caused by the JNK3 pathway.

a) Inactivated JNK3 is bound to scaffold polypeptides JSAP1a, b, c and d which are selectively expressed in the brain.

b) When the JNK3 pathway is activated (MAPKKK → MAPKK → JNK3) by various extracellular stresses, such as THF- $\alpha$ ,

IL-1, EGF, LPS and the like, JNK3 per se is activated (phosphorylated), and the activated JNK3 phosphorylates JSAP1a, b, c and d.

c) After the phosphorylation of JSAP1, JNK3 is released from JSAP1 and transferred into the nucleus.

It is believed that the JNK3 transferred into the nucleus activates various transcription factors and leads the cell into apoptosis and the like. Thus, a compound having an inhibitory activity on phosphorylation of JSAP1 by this JNK3 can inhibit the JNK3 pathway and therefore is useful in preventing and treating a disease caused by the JNK3 pathway, such as apoptosis or the like.

A method for screening a compound having an inhibitory activity on phosphorylation of JSAP1 by JNK3 is described below in detail.

#### 1) Screening system (cell-free system)

##### 1)-1 Screening system 1 (method using radioisotope)

A screening test solution is prepared by adding (i) GST-JSAP1 prepared according to the method described in <4>(1) or JSAP1 and (ii) the activated JNK3 prepared according to the method described in <4>(2) or activated JNK3 and a test compound to a buffer, such as a buffer A (20 mM HEPES, 10 mM  $MgCl_2$ , 5 mM  $\beta$ -mercaptoethanol, 0.1 mg/ml BSA (pH 7.4)) and mixing them. The screening test solution is added to a 96 well plate.

After the addition, [ $\gamma$ - $^{32}\text{P}$ ]ATP or [ $\gamma$ - $^{33}\text{P}$ ]ATP is added to the plate. After the reaction for 5 to 30 minutes, 50 mM EDTA is added to stop the reaction.

After termination of the reaction, the polypeptide in the reaction solution is trapped by vacuum filtration on the membrane of each well of a 96 well nitrocellulose membrane plate, a 96 well phosphocellulose membrane plate or a 96 well PVDF membrane plate, and the membrane is washed by aspiration with the same buffer.

The membrane is put into a liquid scintillator MicroScint-20 (manufactured by Packard) and the  $^{32}\text{P}$  or  $^{33}\text{P}$  radioactivity on the membrane is counted using Top Count (manufactured by Packard).

By the method, a compound having an inhibitory activity on phosphorylation of JSAP1 by JNK3 can be detected using as a control the count of a case in which the test compound is not added.

#### 1)-2 Screening system 2 (ELISA)

An antibody (first antibody) which recognizes JSAP1 obtained according to the method described in <3> is coated on a 96 well plate.

To a screening test solution prepared according to the method described in 1)-1, 50  $\mu\text{M}$  of ATP is added.

After the addition, the reaction is carried out at room temperature for 5 to 60 minutes, and the reaction is terminated with 0.25 N HCl.

After the termination, the reaction solution is neutralized with a solution containing 0.25 N NaOH and 0.1 M Tris-HCl (pH 8.0).

An aliquot of the neutralized solution is added to the above plate coated with the first antibody and allowed to stand.

After the standing, the plate is washed with the same buffer, and then an antibody (second antibody) which recognizes the phosphorylated JSAP1 obtained according to the method described in <3> is added thereto.

By determining the amount of the second antibody by ELISA in a known method, the amount of the phosphorylated JSAP1 is determined.

By the method, a compound having an inhibitory activity on phosphorylation of JSAP1 by JNK3 can be detected using as a control the amount of phosphorylated JSAP1 in a case in which the test compound is not added.

Although the compound which inhibits binding of the polypeptide of the present invention with JNK3 or the compound having the inhibitory activity on phosphorylation of JSAP1, obtained by the methods in the above 1)-1 and 1)-2, can be used directly as a therapeutic agent, generally, it is preferred to use the compound as a pharmaceutical

preparation produced by a well-known method in the technical field of pharmaceuticals by mixing it with at least one pharmaceutically acceptable carrier.

As the administration method of the therapeutic agent, it is preferred to use the most effective method in carrying out the treatment, and methods by oral administration or by parenteral administration, such as buccal, airway, rectal, subcutaneous, intramuscular, intravenous or the like, can be used.

Examples of dosage form of the therapeutic drug include ointments, sprays, capsules, tablets, granules, syrups, emulsions, suppositories injections, tapes and the like.

Examples of preparations suitable for oral administration include emulsions, syrups, capsules, tablets, powders, granules and the like.

Liquid preparations such as emulsions and syrups can be produced using, as additives, water; saccharides, such as sucrose, sorbitol, fructose, etc.; glycols, such as polyethylene glycol, propylene glycol, etc.; oils, such as sesame oil, olive oil, soybean oil, etc.; antiseptics, such as p-hydroxybenzoates, etc.; flavors, such as strawberry flavor, peppermint, etc.; and the like.

Capsules, tablets, powders, granules and the like can be produced using, as additive, fillers, such as lactose, glucose, sucrose, mannitol, etc.; disintegrants,

such as starch, sodium alginate, etc.; lubricants, such as magnesium stearate, talc, etc.; binders, such as polyvinyl alcohol, hydroxypropylcellulose, gelatin, etc.; surfactants, such as fatty acid ester, etc.; plasticizers, such as glycerin, etc.; and the like.

Examples of the preparations suitable for parenteral administration include injections, suppositories, sprays and the like.

The injections can be prepared using, for example, a carrier comprising a salt solution, a glucose solution, or a mixture of both or the like.

The suppositories can be produced using, for example, a carrier, such as cacao butter, hydrogenated fat, carboxylic acid or the like.

As the sprays, the phosphorylation inhibitor or binding inhibitor obtained in the foregoing can be used directly as sprays, but sprays prepared using a carrier or the like which does not stimulate buccal and airway mucous membranes of the recipient and can facilitate absorption of the compound by dispersing it as fine particles are preferred.

Examples of the carrier include lactose, glycerol and the like.

Depending on the properties of the agonist or antagonist obtained in the foregoing and of the carrier, it

is possible to prepare pharmaceutical preparations, such as aerosols, dry powders and the like.

The components exemplified as additive agents of oral preparations can also be added to these parenteral preparations.

The dose or frequency of administration varies depending on the intended therapeutic effect, administration method, treating period, age, body weight and the like, but is generally from 10  $\mu\text{g/kg}$  to 8  $\text{mg/kg}$  per day and per adult.

<5> Screening and identification of compound which controls expression of the polypeptide of the present invention (hereinafter referred to as "expression-controlling compound")

(1) Screening and identification of expression-controlling compound using the antibody of the present invention

After allowing a cell expressing the polypeptide of the present invention to contact with a test sample, an expression-controlling compound existing in the cell or a cell culture supernatant can be screened and identified using the antibody of the present invention.

As the cell, any one of cells, cell lines and tissues which express the polypeptide of the present invention can be used.



Also, a cell, a cell line or tissue in which expression of the polypeptide was observed using the method for the immunological detection with the antibody described in <3> can be used.

As a suitable cell line, a mouse-derived P19 cell (ATCC CRL-1825) which is differentiated to be a neuronal cell like with retinoic acid is exemplified.

As the test sample, those which were exemplified in <4> above can be used.

Cells expressing the polypeptide of the present invention are suspended in a medium in which the cells can grow, each test sample is added to the medium for contact with the cells, and then the amount of the polypeptide expressed in the cells is determined using the antibody of the present invention. As the determination method using the antibody, the immunocytochemistry described below is exemplified.

Cultured adherent cells are washed with PBS, 3 ml of PBS containing 0.05% of trypsin and 0.02% of ethylenediaminetetraacetic acid (EDTA) was added thereto, and then, after removing an extra solution, incubated at 37°C for 5 minutes to peel off the cells from the flask.

The suspension cells can be used as cultured cells, as they are.

Cells used in the immunocytochemistry are suspended in a buffer for immunocytochemistry (PBS containing 1% BSA,

0.02% EDTA and 0.05% sodium azide) or the like and dispensed into a round bottom 96-well plate at  $1 \times 10^5$  to  $20 \times 10^5$  cells per well.

The monoclonal antibody of the present invention is dispensed into the plate.

As the monoclonal antibody, culture supernatant of the hybridoma obtained in <3>(2-3) capable of producing the monoclonal antibody of the present invention and the purified monoclonal antibody obtained in <3>(2-4) can be exemplified. Additionally, an antibody prepared by labeling the monoclonal antibody can be used.

As the antibody prepared by labeling the monoclonal antibody, a biotin-labeled antibody can be exemplified.

The biotin-labeled antibody can be prepared by a known method (*Enzyme Antibody Method*, published by Gakusai Kikaku (1985)).

The above antibody is diluted to a concentration of 0.1 to 50  $\mu\text{g/ml}$  using the buffer for immunocytochemistry or the buffer for immunocytochemistry containing 10% animal serum.

The diluted antibody is dispensed to give an amount of 20 to 500  $\mu\text{l/well}$  and incubated on ice for 30 minutes.

When the unlabeled antibody is used, the cells are washed by adding the buffer for immunocytochemistry to the above plate, the buffer for immunocytochemistry containing about 0.1 to 50  $\mu\text{g/ml}$  of an anti-mouse immunoglobulin

antibody or anti-rat immunoglobulin antibody labeled with fluorescence dye, such as fluorescein isothiocyanate (FITC), phycoerythrin or the like, is dispensed at about 50 to 500  $\mu$ l/well, and then the cells are incubated on ice in the dark for 30 minutes.

When the monoclonal antibody labeled with biotin is used, streptoavidin labeled with a fluorescence dye, such as FITC, phycoerythrin or the like, is dispensed at about 50 to 500  $\mu$ l/well into the above plate, and then incubated on ice in the dark for 30 minutes.

In both cases, after incubation, the cells are thoroughly washed by adding the buffer for immunocytochemistry to the plate, and then analyzed by a fluorescence microscopy, a cell sorter or the like.

The analyzed result obtained by carrying out the same procedure without adding test samples is compared with the analyzed result obtained by adding test samples, and a test sample capable of increasing or decreasing the amount of the polypeptide of the present invention is screened and thereby identified as an expression-controlling compound.

(2) Screening and identification using transcription product determination system of the gene of the polypeptide of the present invention

An expression-controlling compound can be screened and identified by allowing cells which express the

polypeptide of the present invention or mRNA encoding the polypeptide to contact with each test sample, and then determining the amount of the mRNA.

Cells which express the polypeptide of the present invention or mRNA encoding the polypeptide are suspended in a medium in which the cells can grow, each test sample is added to the medium for contact with the cells, and then the amount of the mRNA expressed by the cells is determined using a known Northern hybridization, RNA dot blot hybridization, RT-PCR or the like.

As probes which can be used in the hybridization and the like and primers which can be used in the RT-PCR and the like, fragments of the gene which encode the polypeptide of the present invention can be exemplified.

Specifically, an oligonucleotide having a sequence identical to continuous 5 to 60 bases in the nucleotide sequence represented by SEQ ID NOS:1 to 8 or an oligonucleotide having a sequence complementary to the oligonucleotide can be preferably used.

The determination result obtained by carrying out the same procedure without adding test samples is compared with the determination result obtained by adding test samples, and a test sample capable of increasing or decreasing amount of mRNA encoding the polypeptide of the present invention is screened and thereby identified as an expression-controlling compound.

### (3) Screening and identification using reporter gene

A cell transformed with a plasmid containing a DNA in which a reporter gene is connected to the downstream of a region which controls transcription of a gene which encodes the polypeptide of the present invention (hereinafter referred to as "transcription-controlling region") is allowed to contact with each test sample, and then an expression-controlling compound can be screened and identified by determining the amount of the expressed polypeptide encoded by the reporter gene.

In general, the transcription-controlling region is contained in the 5' upstream of genes in most cases. The 5' upstream region of the gene encoding the polypeptide of the present invention can be prepared by, for example, using Genome Walker kits (manufactured by Clontech) or the like. Also, a fragment obtained by digesting the region into a suitable length using suitable restriction enzymes can be used as the transcription-controlling region.

Any gene can be used as the reporter gene, so long as a translation product of the gene is stable inside the cells and the existing amount of the translation product can easily be determined. Examples include chloramphenicol acetyltransferase (CAT),  $\beta$ -galactosidase ( $\beta$ -gal), luciferase (luc), green fluorescent protein (GFP) and the like.

As the host cell into which a reporter plasmid containing the transcription-controlling region is introduced, any cell can be used; however, a cell line in which expression of the polypeptide of the present invention or mRNA encoding the polypeptide is confirmed as described in <5>(1) is preferably used.

As the test samples, those described in <4> above can be used.

The host cell is transformed by a known method using a plasmid prepared by connecting the reporter gene to the downstream of the transcription-controlling region by a known method.

Alternatively, a cell line in which a part of the gene encoding the polypeptide of the present invention is substituted with a reporter gene can be obtained by preparing a gene targeting vector in which a positive selection marker (G418 resistance gene or the like) and a negative selection marker (thymidine kinase gene of herpes simplex virus, diphtheria toxin A fragment gene or the like) are connected (*Nature*, 336: 348 (1988); *Analytical Biochemistry*, 214: 77 (1993); *Gene Targeting, The Practical Approach Series*, IRL Press (1993)).

The transformant cells are suspended, for example, in a medium in which the cells can grow, each test sample is added to the medium for contact with the cells, and then the amount of the polypeptide encoded by the reporter gene

expressed by the cells is detected and determined using a method suitable for the polypeptide.

Examples of the detection and determination method include the method described in, for example, *Molecular Cloning*, 2nd ed., Chapter 16, p. 60 in the case of CAT; the method described in, for example, *Molecular Cloning*, 2nd ed., Chapter 16, p. 66 in the case of  $\beta$ -gal; the method described in, for example, *Experimental Medicine, Supplement, Biomanual Series 4, Gene Introduction and Expression Analysis Method*, 81 (1994) in the case of luc; the method described in, for example, *Proc. Natl. Acad. Sci. USA*, 94: 4653 (1997) in the case of GFP; and the like.

The determination result obtained by carrying out the same procedure without adding test samples is compared with the determination result obtained by adding test samples, and a test sample capable of increasing or decreasing amount of the polypeptide encoded by the reporter gene is searched and thereby identified as an expression-controlling compound.

<6> Use of the DNA, polypeptide, antibody, binding inhibitor, phosphorylation inhibitor and expression-controlling compound of the present invention

(1) The DNA of the present invention can be used for detecting or determining mRNA of the gene of the polypeptide of the present invention in human tissues and

human-derived cells by carrying out Northern hybridization of RNA extracted from the tissues and cells in the same manner as described in <1>(1) using the DNA as the probe. Expression distribution of the polypeptide of the present invention in tissues can be known by comparing the expression amounts of mRNA in various tissues.

(2) The oligonucleotide of the present invention can be used for detecting and determining mRNA encoding the polypeptide of the present invention by carrying out RT-PCR (reverse transcription PCR; *PCR Protocols* (1990)) of RNA extracted from human tissues and human-derived cells in the same manner as described in <1>(1) using the oligonucleotide of the present invention as a specific primer of the DNA of the present invention.

The method for determining mRNA can be used in the diagnosis of disease states related to the gene.

Importance of the gene product in disease states can be revealed by determining the mRNA in animal models of various disease states. Also, medicaments can be evaluated by comparing expression amounts of the mRNA in the presence or absence of each medicament.

(3) More definite expression distribution, such as specification of the expressing cells of the polypeptide of the present invention in tissues, can be known by carrying



out *in situ* hybridization (*Methods in Enzymology*, 254: 419 (1995)) of tissue sections of human using the oligonucleotide of the present invention as the probe.

Information concerning which tissues and cells express the polypeptide of the present invention and information concerning what kind of stimulation changes the expression level in cells are useful in analyzing participation of the polypeptide of the present invention in physiological functions and disease states.

(4) Mutation of the gene encoding the polypeptide of the present invention can be detected by carrying out Southern hybridization (*Molecular Cloning*, 2nd ed.) on genomic DNA using the DNA of the present invention as the probe.

Neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis and the like, amyotrophic diseases, such as amyotrophic lateral sclerosis and the like, ischemic diseases, brain damage due to stroke, schizophrenia, depression, epilepsy, or various immunological and inflammatory diseases, which have a possibility that mutation of the gene is a cause thereof, can be diagnosed by detecting the mutation.

(5) Neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis and the like, amyotrophic diseases, such as amyotrophic lateral sclerosis and the like, ischemic diseases, brain damage due to stroke, schizophrenia, depression, epilepsy, or various immunological and inflammatory diseases, in which the gene encoding the polypeptide of the present invention is involved in the onset of diseases, can be prevented or treated by inhibiting transcription of the gene encoding the polypeptide of the present invention or translation of the mRNA (*Chemistry*, 46: 681 (1991); *Bio/Technology*, 9: 358 (1992)) using the antisense oligonucleotide (RNA/DNA) of the present invention.

The above antisense oligonucleotide is administered to the living body by designing and preparing it based on an oligonucleotide having a sequence complementary to continuous 5 to 60 bases in the nucleotide sequence of the DNA of SEQ ID NOS:1 to 8 encoding the polypeptide of the present invention, preferably a nucleotide sequence complementary to 5 to 60 bases existing in the translation initiation region of the DNA encoding the polypeptide of the present invention.

The medicament containing the DNA of the present invention can be prepared using a method similar to the preparation method of pharmaceutical preparations of the

phosphorylation inhibitor or binding inhibitor of the polypeptide of the present invention described in <4> above, and the thus prepared pharmaceutical preparations can be administered in the same manner as in <4> above.

(6) The polypeptide of the present invention can be obtained by the method described in <2> using the DNA of the present invention.

An agent for treating or preventing neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis and the like, amyotrophic diseases, such as amyotrophic lateral sclerosis and the like, ischemic diseases, brain damage due to stroke, schizophrenia, depression, epilepsy, or various immunological and inflammatory diseases is considered as the use of the polypeptide of the present invention.

The medicament containing the polypeptide of the present invention can be prepared using a method similar to the preparation method of pharmaceutical preparations of the phosphorylation inhibitor or binding inhibitor of the polypeptide of the present invention described in <4> above, and the thus prepared pharmaceutical preparations can be administered in the same manner as in <4> above.

(7) The oligonucleotide of the present invention can be used in the gene therapy as a vector for gene therapy by inserting it, as a single-stranded or double-stranded chain, into viral vector, such as retrovirus, adenovirus, adeno-associated virus or the like, or other vector.

(8) An antibody for the polypeptide of the present invention can be produced by the method described in <3> using the polypeptide of the present invention as the antigen.

The polypeptide of the present invention can be detected or determined immunologically using the antibody for the polypeptide of the present invention.

Examples include detection methods, such as ELISA using a microtiter plate, immunocytochemistry by enzyme-labeled antibody method or fluorescent antibody method, Western blotting method and the like.

Examples include sandwich ELISA using two monoclonal antibodies having different epitopes, among antibodies which react with the polypeptide of the present invention in solution, and a radioimmunoassay using an antibody capable of recognizing the polypeptide of the present invention labeled with a radioisotope, such as <sup>125</sup>I or the like, and the polypeptide of the present invention.

The antibody of the present invention can also be applied to immunohistochemistry using pathologic tissue sections.

The polypeptide of the present invention existing in cells or tissues of a normal person and a patient is immunologically detected or determined using the antibody of the present invention, the amounts between the normal person and patient are compared, and then a difference in expression level is determined to diagnose neurodegenerative diseases (Alzheimer's disease, Parkinson's disease and the like), ischemic diseases, brain damage due to stroke, epilepsy, or various immunological and inflammatory diseases of patients.

(9) Neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis and the like, amyotrophic diseases, such as amyotrophic lateral sclerosis and the like, ischemic diseases, brain damage due to stroke, schizophrenia, depression, epilepsy, or various immunological and inflammatory diseases can be prevented or treated by administering an antibody which inhibits a function of the polypeptide of the present invention (JNK3 scaffold polypeptide or substrate of phosphorylation by JNK3, or binding with JNK3).

The medicament containing the antibody of the present invention can be prepared using a method similar to the preparation method of pharmaceutical preparations of the phosphorylation inhibitor or binding inhibitor of the polypeptide of the present invention described in <4> above, and the thus prepared pharmaceutical preparations can be administered in the same manner as in <4> above.

(10) The phosphorylation inhibitor and binding inhibitor of the present invention and the compound which controls expression of the gene of the polypeptide of the present invention can be used for preventing or treating neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis and the like, amyotrophic diseases, such as amyotrophic lateral sclerosis and the like, ischemic diseases, brain damage due to stroke, schizophrenia, depression, epilepsy, or various immunological and inflammatory diseases.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a restriction enzyme map of pCDNA3-S-JSAP1a-Δ1.

Fig. 2 is a restriction enzyme map of pCDNA3-S-JSAP1a-Δ2.

Fig. 3 is a restriction enzyme map of pcDNA3-S-JSAP1a-Δ3.

Fig. 4 is a restriction enzyme map of pcDNA3-S-JSAP1a-Δ4.

Fig. 5 is a restriction enzyme map of pcDNA3-S-JSAP1a-Δ5.

Fig. 6 is a restriction enzyme maps of pcDNA3-S-JSAP1a to pcDNA3-S-JSAP1d.

Fig. 7 is a restriction enzyme map of pcDNA3-S-JSAP3.

Fig. 8 is a restriction enzyme map of pcDNA3-S-JSAP4.

Fig. 9 is a restriction enzyme map of pGAD10-JSAP5.

Fig. 10 is a restriction enzyme map of pcDNA3-His-S-JSAP5.

Fig. 11 is an electrophoresis pattern showing a result of Northern hybridization for mRNA of the mouse liver, spleen, kidney, brain, heart, lung and testis using a partial sequence of each cDNA of mouse JNK3 and mouse JSAP1a as the probe. A result on  $\beta$ -actin is also shown as an expression control.

Fig. 12 is an electrophoresis pattern showing a result of test on the binding ability of JSAP1a to JNK1, JNK2, JNK3, ERK2 and p38 analyzed by Western blotting. Lower column shows a result of Western blotting carried out using a cell extract in order to confirm the expression

amount of each of Flag-JNK1, 2, 3, ERK2 and p38 in COS-7 cells.

Fig. 13 is an electrophoresis pattern showing a result of the analysis of JNK3 binding region in JSAP1a.

Fig. 14 is an electrophoresis pattern showing a result of the phosphorylation of JSAP1a by JNK3.

Fig. 15 is an electrophoresis pattern showing a result of the analysis of the phosphorylation of JSAP1a and intracellular localization of JNK3. JNK3 was detected by antibody staining. The cell nucleus was detected by Hoechst staining.

Fig. 16 is an electrophoresis pattern showing a result of Western blotting analysis on the binding ability of JSAP1a to SEK1. Lower two columns show a result of Western blotting carried out using a cell extract in order to confirm the expression level of each Flag-SEK1 and activated SEK1 in COS-7 cells. FL means a full length polypeptide.

Fig. 17 is an electrophoresis pattern showing a result of Western blotting analysis on the binding ability of JSAP1a to MKK7. Lower column shows a result of Western blotting carried out using a cell extract in order to confirm the expression level of each Flag-MKK7 in COS-7 cells. FL means a full length polypeptide.

Fig. 18 is an electrophoresis pattern showing a result of Western blotting analysis on the binding ability



of JSAP1a to MEK1, MKK6 and MKK7. Lower column shows a result of Western blotting carried out using a cell extract in order to confirm the expression amount of each of Flag-MEK1, MKK6 and MKK7 in COS-7 cells. FL means a full length polypeptide.

Fig. 19 is an electrophoresis pattern showing a result of Western blotting analysis on the binding ability of JSAP1a to an N-terminal moiety (1st to 640th amino acid residues) of MEKK1. Lower column shows a result of Western blotting carried out using a cell extract in order to confirm the expression amount of the N-terminal moiety of each Flag-MEKK1 in COS-7 cells. FL means a full length polypeptide.

Fig. 20 is an electrophoresis pattern showing a result of Western blotting analysis on the binding ability of JSAP1a to an N-terminal moiety (1st to 327th amino acid residues) or C-terminal moiety (316th to 648th amino acid residues) of Flag-c-Raf1. Lower column shows a result of Western blotting carried out using a cell extract in order to confirm the expression level of each of Flag-Raf-N and Raf-C in COS-7 cells. FL means a full length polypeptide.

Fig. 21 shows a result of examination on the effects of Cdc42 and JSAP1a on the JNK3 activity, carried out using a LUC reporter system in which the JNK3 activity in P19 cells is measured as the GAL4-c-Jun transcription

activity. The longitudinal axis shows a relative luciferase activity corresponding to the JNK3 activity.

Fig. 22 shows a result of examination on the effects of  $\Delta$ Raf1 and JSAP1a on the ERK activity, carried out using a LUC reporter system in which the ERK activity in P19 cells is measured as the GAL4-Elk1 transcription activity. The longitudinal axis shows a relative luciferase activity corresponding to the ERK activity.

Fig. 23 is an electrophoresis pattern showing a result of Western blotting analysis, carried out using a  $\Delta$ MEKK1 enzyme solution, a JNK3 enzyme solution and an activated JNK3 enzyme solution obtained by introducing  $\Delta$ MEKK1 and JNK3 genes in COS-7 cells singly or simultaneously. "A" shows a result of staining of activated JNK3 with an antibody which recognizes phosphorylated JNK3, and "B" shows a result of staining of JNK3 with anti-Flag antibody. The  $\Delta$ MEKK1 enzyme solution was applied to lane 1 of the electrophoresis, and the activated JNK3 enzyme solution to lane 2, the JNK3 enzyme solution to lane 3 and a disrupted solution of COS-7 cells without gene introduction to lane 4.

Fig. 24 is an electrophoresis pattern showing a result of Western blotting analysis on the binding ability of JSAP3, 4 and 5 to JNK3. Lower column shows a result of Western blotting carried out using a cell extract in order

to confirm the expression amount of each of Flag-JNK3 in COS-7 cells.

Fig. 25 is an electrophoresis pattern showing a result of autoradiography on the binding ability of JSAP3 to ATF2.

Fig. 26 is an electrophoresis pattern showing a result of autoradiography on the expression of <sup>35</sup>S-labeled JSAP4 molecules having various length.

Fig. 27 is an electrophoresis pattern showing a result of autoradiography of the JNK3 binding region of JSAP4. This is a result of the analysis of the binding of <sup>35</sup>S-labeled JSAP4 molecules having various length with GST or GST-JNK3, and it is observed as a band.

Fig. 28 is an electrophoresis pattern showing a result of Western blotting analysis on the binding ability of JSAP4 to JNK1, JNK2 and ERK2. Among the three columns of blotting, the second and third columns show results of Western blotting carried out using cell extracts in order to confirm the expression amounts of JSAP4 and each MAPK (JNK1, JNK2, JNK3 and ERK2), respectively, in COS-7 cells.

Fig. 29 shows a result of Northern hybridization carried out on mRNA of the mouse testis, large intestine, heart, lung, kidney, brain, spleen and liver using a partial sequence of cDNA of JSAP4 as the probe. A result on  $\beta$ -actin is also shown as an expression control.

Fig. 30 shows a result of examination on the effects of MEKK1, TAK1 and JSAP4 on the JNK activity, carried out using a LUC reporter system in which the JNK activity in COS-7 cells is measured as the GAL4-c-Jun transcription activity. The longitudinal axis shows a relative luciferase activity corresponding to the JNK activity.

Fig. 31 shows a result of examination on the effects of  $\Delta$ Raf1 and JSAP4 on the ERK activity, carried out using a LUC reporter system in which the ERK activity in COS-7 cells is measured as the GAL4-Elk1 transcription activity. The longitudinal axis shows a relative luciferase activity corresponding to the ERK activity.

Description of the Symbols:

kb: kilobase pairs

Ap: ampicillin-resistant gene

knt: kilonucleotides

#### BEST MODE FOR CARRYING OUT THE INVENTION

The present invention is explained based on the examples, but the scope of the present invention is not limited thereto.

Unless otherwise noted, the known methods described in *Molecular Cloning*, 2nd ed. were used as the gene manipulation techniques in the following examples.

Example 1 Cloning of cDNA encoding a polypeptide having the activity to bind to JNK3

(1) Cloning from mouse brain-derived cDNA library

A full length cDNA encoding the mouse JNK3 (*Nature Medicine*, 3: 89 (1997)) was inserted into the *NcoI*-*BamHI* site of cloning vector pAS2-1 (manufactured by Clontech) containing a sequence encoding GAL4 DNA binding domain (pAS2-1-JNK3), and then introduced into yeast CG-1945 (manufactured by Clontech).

A mouse brain cDNA library in cloning vector pGAD10 (manufactured by Clontech) containing a sequence encoding the GAL4 transcription activation domain was introduced into the above yeast which had been introduced with pAS2-1-JNK3 to obtain yeast transformants.

Strains from which the histidine requirement was removed (capable of growing on a histidine-free medium) or strains having  $\beta$ -galactosidase activity were selected from the yeast transformants (primary positive clones).

Clones having no histidine requirement and having  $\beta$ -galactosidase activity were selected from the resulting primary positive clones (secondary positive clones).

Plasmid derived from pGAD10 was recovered from the resulting clones.

As a result, four kinds of partial length cDNA fragments having different sequences were obtained from the

mouse brain cDNA library by a two-hybrid system using the above yeast.

Using these obtained fragments as probes, a  $\lambda$ ZAPII mouse brain cDNA library (manufactured by Clontech) was screened in a known method, and seven kinds of cDNA clones encoding JSAP1a, JSAP1b, JSAP1c, JSAP1d, JSAP3, JSAP4 and JSAP5 as polypeptides capable of binding to JNK3 (JSAP) were obtained.

The cDNA encoding JSAP1a, JSAP1b, JSAP1c, JSAP1d, JSAP3 or JSAP4 was obtained as a full length cDNA, but the cDNA encoding JSAP5 was obtained as a partial length cDNA.

It was found that the cDNA encoding JSAP1a, JSAP1b, JSAP1c, JSAP1d, JSAP3 or JSAP4 contained an open reading frame (hereinafter referred to as "ORF") of 3,918 bp, 3,945 bp, 4,014 bp, 4,011 bp, 1,293 bp or 4,527 bp, respectively, which encoded amino acid residues of 1305 residues, 1314 residues, 1337 residues, 1336 residues or 1508 residues, respectively (SEQ ID NOS:1 to 6).

Also, JSAP1a, JSAP1b, JSAP1c and JSAP1d were splice variants derived from the same gene. That is, a variant in which a nucleotide sequence of 27 bp had been inserted into JSAP1a was JSAP1b, a variant in which nucleotide sequences of 3 bp and 93 bp were inserted was JSAP1c, and a variant in which a nucleotide sequence of 93 bp was inserted was JSAP1d.

Specifically, the insertion site of JSAP1b is a 27 bp DNA sequence moiety encoding 9 amino acid residues from the 201st serine residue to the 209th serine residue of SEQ ID NO:10; the insertion sites of JSAP1c are a 3 bp DNA sequence moiety encoding the 201st serine residue and a 93 bp DNA sequence moiety encoding 31 amino acid residues from the 219th valine residue to the 249th glutamine residue of SEQ ID NO:11; and the insertion site of JSAP1d is a 93 bp DNA sequence moiety encoding 31 amino acid residues from the 218th valine residue to the 248th glutamine residue of SEQ ID NO:12.

JSAP1a coincided with a known sequence (SEQ ID NO:9, 1997 The Molecular Biology Society of Japan (December)).

It was estimated that JSAP3 is a mouse homologue of human C-terminal-binding protein 1 (CtBP) (*EMBO J.*, 17: 5129 (1998)) (SEQ ID NO:13).

In JSAP4 having the amino acid sequence represented by SEQ ID NO:14, a sequence called WD40-repeat was found in the 87th to 121st positions, the 341st to 373rd positions, the 658th to 690th positions and the 700th to 732nd positions of the amino acid sequence.

It is expected that the sequence can be concerned in an interaction (binding) with other protein, and it can be found in a molecule which takes part in the intracellular signal transduction, such as G-protein (*FEBS Lett.*, 307: 131 (1994)). Based on Examples described below,

it was revealed that the sequence has an important function on signal transduction in the JNK3 pathway.

The cDNA encoding JSAP5 was a DNA constituted by 734 bp shown in SEQ ID NO:7. It was found that this cDNA does not contain initiation and termination codons and therefore is a partial length. The cDNA encoded 244 amino acid residues (SEQ ID NO:15).

Full length cDNA of JSAP5 (SEQ ID NO:8) was obtained from the  $\lambda$ ZAPII mouse brain cDNA library (manufactured by Clontech) by a known method using a probe prepared based on the information on the cDNA nucleotide sequence of JSAP5. It was found that the cDNA contains an ORF of 1,455 bp and encodes 484 amino acid residues (SEQ ID NO:16, hereinafter referred to as "JSAP5F"). In the following experiment, cDNA of JSAP5 as a partial length cDNA of JSAP5F was used.

(2) Preparation of various polypeptides and vectors expressing the polypeptides for analyzing properties of JSAP

In order to analyze functions of the resulting polypeptide JSAP capable of binding to JNK3, the following polypeptides and vectors which express the polypeptides were prepared.



1) Preparation of vectors which express fusion polypeptides with thioredoxin•S-tag (hereinafter referred to as "Trx•S") peptide

By a known method, cDNA samples of JNK1, JNK2, JNK3 and SEK1 were obtained from a  $\lambda$ ZAPII mouse brain cDNA library (manufactured by Clontech), and the MEKK1 cDNA from a  $\lambda$ ZAPII mouse spleen cDNA library (manufactured by Clontech).

The c-Raf1 cDNA was obtained from a cDNA library provided by Health Science Research Resources Bank, Japan.

Samples of cDNA of human lymphocyte-derived MKK6, p38, ERK2, c-Jun(1-79) and ATF2 and mouse thymus-derived MEK1, MKK7 and Cdc42 were obtained by using a PCR method.

Each of the resulting cDNA was inserted into the downstream of the Trx•S sequence of pET32a (manufactured by Novagen) to prepare a respective vector which expresses Trx•S-JNK1, Trx•S-JNK2, Trx•S-JNK3, Trx•S-SEK1, Trx•S-MEKK1, Trx•S-c-Raf1, Trx•S-MKK6, Trx•S-p38, Trx•S-ERK2, Trx•S-c-Jun, Trx•S-ATF2, Trx•S-MEK1, Trx•S-MKK7 or Trx•S-Cdc42.

A DNA encoding the JSAP obtained in the above step (1) or a fragment of the DNA was inserted into each of the restriction enzyme sites described below existing in the downstream of the Trx•S sequence of expression vector pET32a (manufactured by Novagen) to prepare a respective vector which expresses Trx•S-JSAP1a, Trx•S-JSAP1b, Trx•S-

JSAP1c, Trx•S-JSAP1d, Trx•S-JSAP3, Trx•S-JSAP4 or Trx•S-JSAP5.

Polypeptide encoded by each DNA inserted and each restriction enzyme site for insertion are shown below.

JSAP1a (115th to 274th amino acid residues):

*NcoI*-*BamHI* site

JSAP1a (115th to 504th amino acid residues):

*EcoRI* site

JSAP1a (268th to 486th amino acid residues):

*NcoI* site

JSAP1a (486th to 744th amino acid residues):

*NcoI*-*BamHI* site

JSAP1a (744th to 1194th amino acid residues):

*BamHI* site

JSAP1b (115th to 283rd amino acid residues):

*NcoI*-*BamHI* site

JSAP1c (115th to 306th amino acid residues):

*NcoI*-*BamHI* site

JSAP1d (115th to 305th amino acid residues):

*NcoI*-*BamHI* site

JSAP3 (full length):

*EcoRI*-*SalI* site

JSAP4 (1042nd to 1331st amino acid residues):

*EcoRI*-*HindIII* site

JSAP5 (partial length):

*EcoRI* site

Also, Trx•S-ATF2 (1st to 107th amino acid residues) and Trx•S-ATF2 (1st to 116th amino acid residues) were prepared by inserting a DNA encoding 1st to 107th amino acid residues of ATF2 and a DNA encoding 1st to 116th amino acid residues of ATF2 into *Bam*HI-*Xho*I and *Bam*HI-*Xho*I sites, respectively, existing in the downstream of the Trx•S sequence of the expression vector pET32a (manufactured by Novagen).

2) Preparation of vectors which express fusion polypeptides with Flag peptide

Each full length cDNA encoding JNK1, JNK2, JNK3, ERK2 or p38 was inserted into the *Not*I-*Bam*HI site existing in the downstream of the Flag sequence of mammal expression vector pFlag-CMV-2 (manufactured by Kodak) to prepare a corresponding vector which expresses Flag-JNK1, Flag-JNK2, Flag-JNK3, Flag-ERK2 or Flag-p38, respectively.

Also, a DNA encoding each of the following polypeptides was inserted into each of the following restriction enzyme sites existing in the downstream of the Flag sequence of Flag-modified pcDNA3 vector to prepare a respective vector which expresses Flag-SEK1, Flag-MKK6, Flag-MKK7, Flag-MEK1, Flag-MEKK1, Flag-c-Raf1, Flag-Raf-C, Flag-MEKK-N or Flag-TAK1.

Polypeptide encoded by each DNA inserted and each restriction enzyme site for insertion are shown below.

SEK1 (full length):

*HindIII-XbaI* site

MKK6 (full length):

*HindIII-XbaI* site

MKK7 (full length):

*HindIII-XbaI* site

MEK1 (full length):

*HindIII-XbaI* site

MEKK1 (full length):

*BamHI-EcoRV* site

c-Raf1 (full length):

*EcoRI-XhoI* site

Raf-N (1st to 327th amino acid residues):

*EcoRI-EcoRV* site

Raf-C (316th to 648th amino acid residues):

*EcoRV-XhoI* site

MEKK-N (1st to 640th amino acid residues):

*BamHI-EcoRI* site

TAK1 (full length):

*EcoRI-XhoI* site

The DNA encoding TAK1 (TGF- $\beta$ -activated kinase 1) (*Science*, 270: 2008 (1995)) was obtained by a known method from a mouse cell strain BAF-B03 cDNA library.

3) Preparation of vectors which express fusion polypeptides with GST

DNA encoding 1st to 79th amino acid residues of c-Jun into the *Bam*HI-*Eco*RI site of GST fusion protein expression vector pGEX-3X (manufactured by Pharmacia) to prepare GST-c-Jun(1-79) expression vector.

*E. coli* was transformed using the expression vector by a known method to express GST-c-Jun(1-79).

The thus expressed GST-c-Jun(1-79) was purified using Glutathione Sepharose 4B (manufactured by Pharmacia).

*Nco*I (blunt ended)-*Bam*HI (blunt ended) DNA fragment encoding JNK3 (full length) was inserted into the *Bam*HI (blunt ended) site of GST fusion protein expression vector pGEX-2T (manufactured by Pharmacia) to prepare GST-JNK3 expression vector.

*E. coli* was transformed using the expression vector by a known method to prepare GST-JNK3. The thus expressed GST-JNK3 was purified using Glutathione Sepharose 4B (manufactured by Pharmacia).

4) Preparation of vectors which express fusion polypeptides with S-tag peptide

DNA encoding each of the polypeptides described below or a fragment of the DNA was inserted into each of the restriction enzyme sites described below existing in the downstream of the S-tag sequence of expression vector S-

modified pcDNA3 to prepare a respective vector which expresses S-JSAP1a, S-JSAP1a $\Delta$ 1, S-JSAP1a $\Delta$ 2, S-JSAP1a $\Delta$ 3, S-JSAP1a $\Delta$ 4, S-JSAP1a $\Delta$ 5, S-JSAP1b, S-JSAP1c, S-JSAP1d, S-JSAP3, S-JSAP4, S-JSAP4 (1-754), S-JSAP4 (755-1508), S-JSAP4 (755-1062), S-JSAP4 (1063-1331) or S-JSAP4(1332-1508).

Polypeptide encoded by each DNA inserted and each restriction enzyme site for insertion are shown below.

JSAP1a (full length):

*Nco*I site

JSAP1a $\Delta$ 1 (1st to 1053rd amino acid residues):

*Not*I-*Xho*I site

JSAP1a $\Delta$ 2 (744th to 1305th amino acid residues):

*Not*I site

JSAP1a $\Delta$ 3 (1054th to 1305th amino acid residues):

*Bam*HI site

JSAP1a $\Delta$ 4 (343rd to 1053rd amino acid residues):

*Hind*III-*Xho*I site

JSAP1a $\Delta$ 5 (1st to 343rd amino acid residues):

*Hind*III site

JSAP1b (full length):

*Not*I site

JSAP1c (full length):

*Not*I site

JSAP1d (full length):

*Not*I site

JSAP3 (full length):

*EcoRI-XhoI/SalI*

JSAP4 (full length):

*EcoRI-HindIII*

JSAP4 (1st to 754th amino acid residues):

*EcoRI-HindIII*

JSAP4 (755th to 1508th amino acid residues):

*EcoRI-HindIII*

JSAP4 (755th to 1062nd amino acid residues):

*EcoRI-HindIII*

JSAP4 (1063rd to 1331st amino acid residues):

*EcoRI-HindIII*

JSAP4 (1332nd to 1508th amino acid residues):

*EcoRI-HindIII*

Also, cDNA (partial length) of JSAP5 was inserted into the *EcoRI* site existing in the downstream of the GAL4AD sequence of expression vector pGAD10 (manufactured by Clontech), and cDNA (full length) of JSAP5F into the *EcoRV* (blunt ended)-*HindIII* site existing in downstream of the His-S-tag sequence of expression vector His-S-modified pCDNA3 (Figs. 1 to 10).

5) Preparation of vector which expresses JNK3

Plasmid pGEM-NCO in which the *NcoI* site had been added to pGEM-3Zf(+) (manufactured by Promega) was prepared by digesting pGEM-3Zf(+) with *EcoRI* and then carrying out

self-ligation by adding an oligonucleotide linker having a nucleotide sequence of GCCATGC.

JNK3 (full length) was inserted into the *NcoI*-*BamHI* site of the pGEM-NCO to prepare expression vector (pGEM-JNK3).

6) Preparation of vector which expresses constitutively activated Cdc42

Constitutively activated Cdc42 was prepared by converting the 12th glycine of Cdc42 into valine by point mutation (Cdc42(G12V)). A DNA fragment (*BamHI*-blunt end) encoding the Cdc42(G12V) (full length) was inserted into the *BamHI*-*EcoRV* site existing in the downstream of S-tag sequence of the expression vector s-modified pcDNA3.

7) Preparation of vector which expresses constitutively activated MEKK1

cDNA encoding  $\Delta$ MEKK1 (1169th to 1488th amino acid residues; constitutively activated as truncated form of MEKK1) was inserted into the *XbaI* site of pEF-BOS vector (*Nucleic Acids Res.*, 18: 5322 (1990)).



8) 5XGAL4-LUC reporter, GAL4-c-Jun and GAL4-Elk1 expression vectors

All of the 5XGAL4-LUC reporter, GAL4-c-Jun and GAL4-Elk1 expression vectors were purchased from Stratagene and used.

9) RL (*Renilla luciferase*) control vector

The RL control vector was purchased from Promega and used.

10) Preparation of vectors which express fusion polypeptides with Myc-tag peptide

cDNA encoding JSAP4 (full length) or JSAP4 (1063rd to 1331st amino acid residues) was inserted into the *EcoRI*-*NotI* site existing in the downstream of Myc-tag sequence of the expression vector Myc-modified pcDNA3 to prepare a respective vector which expresses Myc-JSAP4 (full length) or Myc-JSAP4 (1063rd to 1331st).

11) Preparation of vectors which express fusion polypeptides with His-S-tag peptide

DNA encoding each of the polypeptides described below was inserted into each of the restriction enzyme sites described below existing in the downstream of the His-S-tag sequence of expression vector His-S-modified pcDNA3 which encodes the His-S-tag to prepare a respective vector

which expresses MAPK-His-S, MAPKKK-His-S, JNK1-His-S, JNK2-His-S, JNK3-His-S, ERK2-His-S or MEKK1-His-S.

The insertion restriction enzyme site of each DNA encoding the full length polypeptide is shown below.

JNK1 (*Not*I (blunt end)-*Bam*HI DNA fragment):

*Eco*RV-*Bam*HI

JNK2 (*Not*I (blunt end)-*Bam*HI DNA fragment):

*Eco*RV-*Bam*HI

JNK3 (*Not*I (blunt end)-*Bam*HI (blunt end) DNA fragment):

*Eco*RV

ERK2 (*Bam*HI DNA fragment):

*Bam*HI

MEKK1 (*Hind*III DNA fragment):

*Hind*III

Example 2 JSAP1a, JSAP1b, JSAP1c and JSAP1d

Since the results of analysis of JSAP1a, JSAP1b, JSAP1c and JSAP1d described below were identical to one another, the results of JSAP1a are shown in the drawings as the representative example. Hereinafter, JSAP1a, JSAP1b, JSAP1c and JSAP1d are collectively to as "JSAP1".

1) Analysis of the expression of JNK3 and JSAP1 mRNA by Northern hybridization

Northern hybridization was carried out according to the method described in *Proc. Natl. Acad. Sci. USA*, 92: 4972 (1995).

Specifically, each of the liver, spleen, kidney, brain, heart, lung and testis of mouse was analyzed using <sup>32</sup>P-labeled JNK3, JSAP1 and  $\beta$ -actin as the probe.

The results are shown in Fig. 11.

Expression of JNK3 was found specifically in brain. With regard to JSAP1a, JSAP1a mRNA having a size of about 6 kb was found in specifically brain.

2) Analysis of binding specificity and binding region of JSAP1 for various MAPK's

Each of the S-JSAP1a, S-JSAP1b, S-JSAP1c or S-JSAP1d (full length) expression vector or the Flag-JNK1, Flag-JNK2, Flag-JNK3, Flag-ERK2 or Flag-p38 expression vector, prepared in Example 1(2), was transfected to COS-7 cells using TransIT-LT1 (manufactured by Mirus), and then the COS-7 cells were cultured to transiently express the polypeptide derived from the respective introduced vectors.

After culturing for 34 hours, the cells were dissolved in buffer B, S Protein Agarose (manufactured by Novagen) was added thereto, and then S-JSAP1 and a

polypeptide capable of binding to S-JSAP1 were precipitated and recovered.

The resulting recovered fraction was separated by SDS-PAGE and transferred on membrane Immobilon-P (manufactured by Millipore).

Western blotting was carried out using the membrane and anti-Flag M5 monoclonal antibody (manufactured by Kodak) as the probe, and the polypeptide capable of binding to the antibody (Flag-JNK3) was visualized using an ECL detection system (manufactured by Amersham) to examine MAPK capable of binding to JSAP1.

The results are shown in Fig. 12. It was found that JSAP1 binds only to JNK3 and does not bind to other MAPK.

The binding region of JSAP1 to JNK3 was analyzed by the following method.

A fusion polypeptide of Trx•S with a partial fragment of JSAP1, each Trx•S-JSAP1 (fragment) was expressed in *E. coli* using the expression vector prepared in Example 1(2) by a known method and then recovered by allowing it to bind to S Protein Agarose.

<sup>35</sup>S-labeled form of full length JNK3 was prepared by *in vitro* translation using the expression vector pGEM-JNK3 prepared in Example 1(2) by TNT T7 Quick Coupled Transcription/Translation System (manufactured by Promega).

The resulting  $^{35}\text{S}$ -labeled JNK3 and Trx•S-JAP1 (fragment) were mixed in buffer A (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40), and the reaction was carried out at 4°C for 2 hours while stirring the reaction solution by rotating its container tube.

After the reaction, the reactant was washed three times with buffer A, and the resulting precipitate was separated by SDS-PAGE and analyzed by autoradiography.

As a result, it was found that the binding region of JSAP1 to JNK3 is present in a region of the 115th to 274th (JSAP1a), 115th to 283rd (JSAP1b), 115th to 306th (JSAP1c) or 115th to 305th (JSAP1d) amino acid residues.

The results in the case of JSAP1a are shown in Fig. 13. In the case of JSAP1a, the binding region was the 115th to 274th amino acid residues.

### 3) Phosphorylation of JSAP1 by JNK3 and loss of binding ability of JNK3 by phosphorylation

In the binding region of JSAP1 with JNK3, threonine residues having a possibility of being phosphorylated by proline-directed serine/threonine kinase are present as shown below.

JSAP1a: 234th, 244th and 255th amino acid residues

JSAP1b: 243rd, 253rd and 264th amino acid residues

JSAP1c: 266th, 276th and 287th amino acid residues

JSAP1d: 265th, 275th and 286th amino acid residues

Fusion polypeptides of each JSAP fragment containing the above positions expected to be phosphorylated with Trx•S, Trx•S-JSAP1a (115th to 274th amino acid residues), Trx•S-JSAP1b (115th to 283rd amino acid residues), Trx•S-JSAP1c (115th to 306th amino acid residues) and Trx•S-JSAP1d (115th to 305th amino acid residues) were prepared according to Example 1(2).

COS-7 cells were transfected using TransIT-LT1 (manufactured by Mirus) with the Flag-JNK3 expression vector and  $\Delta$ MEKK1 expression vector, or the Flag-JNK3 expression vector alone, prepared in Example 1(2), and then the COS-7 cells were cultured to transiently express the polypeptide derived from each of the introduced vectors.

After culturing for 34 hours, the cells were dissolved in buffer B and then Flag-JNK3 was immunoprecipitated using anti-Flag M5 monoclonal antibody (manufactured by Kodak) immobilized on protein-G-agarose.

Using the resulting JNK3 or activated JNK3 and the Trx•S-JSAP1a (115th to 274th amino acid residues), Trx•S-JSAP1b (115th to 283rd amino acid residues), Trx•S-JSAP1c (115th to 306th amino acid residues) or Trx•S-JSAP1d (115th to 305th amino acid residues) prepared above and bound to S protein agarose, phosphorylation was carried out according to the method described in *Cell*, 76: 1025 (1994) by adding  $^{32}\text{P}$ -labeled ATP ( $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ). As a positive control of phosphorylation, GST-c-Jun (1st to 79th amino acid

residues) was used as the substrate (*EMBO J.*, 15: 2760 (1996)).

The reactant was separated by SDS-PAGE and then analyzed by autoradiography.

The results are shown in Fig. 14. The JSAP1 was efficiently phosphorylated (lane 4 in Fig. 14). Phosphorylation of c-Jun as the positive control was also confirmed (lane 2 of Fig. 14).

Each of the above threonine residues having a possibility of being phosphorylated in JSAP1 was converted into an alanine residue by site-specific mutagenesis using overlapping PCR (*Current Protocols in Molecular Biology* (John Wiley & Sons Inc., New York, 1989)), and the converted polypeptide was expressed and recovered according to the method of Example 1(2).

The resulting polypeptides WT to T-3 are shown below.

(i) Trx<sup>S</sup>-JSAP1a (115th to 274th amino acid residue region)

WT: No substitution by an alanine residue

T-0: Substitution with an alanine residue at the 234th, 244th and 255th positions

T-1: Substitution with an alanine residue at the 244th and 255th positions

T-2: Substitution with an alanine residue at the 234th and 255th positions

T-3: Substitution with an alanine residue at the 234 and 244th positions

(ii) Trx•S-JSAP1b (115th to 283rd amino acid residue region)

WT: No substitution by an alanine residue

T-0: Substitution with an alanine residue at the 243rd, 253rd and 264th positions

T-1: Substitution with an alanine residue at the 253rd and 264th positions

T-2: Substitution with an alanine residue at the 243rd and 264th positions

T-3: Substitution with an alanine residue at the 243rd and 253rd positions

(iii) Trx•S-JSAP1c (115th to 306th amino acid residue region)

WT: No substitution by an alanine residue

T-0: Substitution with an alanine residue at the 266th, 276th and 287th positions

T-1: Substitution with an alanine residue at the 276th and 287th positions

T-2: Substitution with an alanine residue at the 266th and 287th positions

T-3: Substitution with an alanine residue at the 266th and 276th positions



(iv) Trx•S-JSAP1d (115th to 305th amino acid residue region)

WT: No substitution by alanine residue

T-0: Substitution with an alanine residue at the 265th, 275th and 286th positions

T-1: Substitution with an alanine residue at the 275 and 286th positions

T-2: Substitution with an alanine residue at the 265 and 286th positions

T-3: Substitution with an alanine residue at the 265 and 275th positions

Phosphorylation was carried out using the resulting polypeptides.

The results in the case of Trx•S-JSAP1a are shown in Fig. 14.

In all of Trx•S-JSAP1a to d, phosphorylation was not observed only in T-0 in which all of the threonine residues expected to undergo phosphorylation were substituted with alanine (lane 5 in Fig. 14), and WT and all of other alanine substitution products (T-1 to 3) were phosphorylated (lanes 6 to 8 in Fig. 14).

According to the method of Example 2(1), the T-0 which were not phosphorylated in the above, Flag-JNK3 and ΔMEKK1 were expressed in P19 cells which were

simultaneously differentiated by retinoic acid, and the cells were stained with anti-Flag M5 monoclonal antibody (manufactured by Kodak).

The same test was carried out using WT which was phosphorylated in the above, Flag-JNK3 and  $\Delta$ MEKK1, and the cells were stained.

The results in the case of JSAP1a are shown in Fig. 15.

In each case of JSAP1, JNK3 was present only in the cytoplasm and unable to transfer into the nucleus when T-0 was used, but JNK3 was transferred into the nucleus when WT was used.

The above results show that, when JSAP1 is phosphorylated by JNK3, the JNK3 bound to JSAP1 is dissociated from JSAP1 and transferred into the nucleus.

#### 4) Binding of various MAPKK and MAPKKK with JSAP1

COS-7 cells were transfected using TransIT-LT1 (manufactured by Mirus) with three kinds of vectors, the S-JSAP1 (full length) expression vector, the expression vector of Flag-SEK1 (Flag-fusion polypeptide of SEK1 (MKK4) as MAPKK), and the  $\Delta$ MEKK1 expression vector, or two kinds of vectors, the S-JSAP1 (full length) expression vector and the Flag-SEK1 expression vector, prepared in Example 1(2), and then the COS-7 cells were cultured to transiently

express the polypeptide derived from each of the introduced vectors.

After culturing for 34 hours, the cells were dissolved in buffer B and then S-JSAP1 and a polypeptide which binds to S-JSAP1 was immunoprecipitated using S-protein agarose (manufactured by Novagen).

The resulting fraction was separated by SDS-PAGE and transferred on membrane Immobilon-P (manufactured by Millipore), and then Flag-SEK1 was visualized by an ECL detection system (manufactured by Amersham) using anti-Flag M5 monoclonal antibody (manufactured by Kodak) as the probe.

The results are shown in Fig. 16. The binding with JSAP1 was observed when SEK1 was activated by  $\Delta$ MEKK1 (lanes 2 and 3 in Fig. 16). The activation of SEK1 by  $\Delta$ MEKK1 was confirmed by Western blotting using a monoclonal antibody which recognizes SEK1 activated by phosphorylation (manufactured by NEB).

Based on the results obtained in the above, the SEK1 binding region in JSAP1 was analyzed in the same manner as the above. That is, various deletion mutants of S-JSAP1 were produced, and binding of each of them to Flag-SEK1 was examined.

The results obtained using deletion mutants derived from JSAP1a are shown in lanes 4 to 8 of Fig. 16. SEK1 was able to bind to the full length FL (1st to 1305th residues), a deletion mutant  $\Delta$ 2 (having 744th to 1305th residues) and

a deletion mutant  $\Delta 3$  (having 1054th to 1305th residues) of JSAP1a (lanes 5, 7 and 8 in Fig. 16), but unable to bind to a deletion mutant  $\Delta 1$  (having 1st to 1053rd residues) (lane 6 in Fig. 16).

Based on the above, it was found that SEK1 binds to the 1054th to 1305th amino acid residues C-terminal portion of JSAP1a.

Binding of other members of MAPKK to JSAP1a was also analyzed in the same manner as the above.

The results are shown in Figs. 17 and 18.

Similar to the case of SEK1, MKK7 (other MAPKK of the JNK pathway) bound to the 1054th to 1305th residues at the C-terminal portion of JSAP1a (Fig. 17). MEK1 and MKK6 as MAPKK of the ERK or p38 pathway, respectively, also bound to JSAP1a (Fig. 18).

COS-7 cells were transfected using TransIT-LT1 (manufactured by Mirus) with the expression vector of S-JSAP1a (full length) FL, a deletion mutant  $\Delta 1$  (having 1st to 1053rd amino acid residues) or a deletion mutant  $\Delta 4$  (having 343rd to 1053rd amino acid residues), and the expression vector of Flag-MEKK-N as a fusion polypeptide of an N-terminal sequence polypeptide of an MEKK1, which is MAPKKK (MEKK1-N: 1st to 640th amino acid residues) with Flag-tag, obtained in Example 1(2), and then the COS-7 cells were cultured for transient expression of the polypeptide derived from each of the introduced vectors.

Since the expression of full length MEKK1 in COS-7 cells was considerably low, MEKK1-N as a partial sequence of MEKK1 was used in this experiment.

The results are shown in Fig. 19. The MEKK1-N bound to each of FL,  $\Delta 1$  and  $\Delta 4$  of JSAP1a. Since it bound to  $\Delta 4$ , it was considered that MEKK1-N binds to the region of 343rd to 1053rd amino acid residues.

The similar experiment was carried out using a polypeptide having a region other than the N-terminal moiety of MEKK1, and it was confirmed that the polypeptide does not bind to JSAP1a at a region other than the above N-terminal moiety.

Since MEKK1 binds to JSAP1a  $\Delta 1$  (1st to 1053rd amino acid residues) having higher affinity than that of the full length JSAP1a (lane 3 in Fig. 19), there is a possibility that the region of 1054th to 1305th residues at the C-terminal of JSAP1a considered in the above to be the binding region of SEK1 has a function of inhibiting the binding of MEKK1.

In addition, among the MAPK pathway, c-Raf1 as a member of MAPKKK involved in the ERK pathway was examined for its binding to JSAP1.

COS-7 cells were transfected with vector expressing Flag-Raf-N or Flag-Raf-C obtained in Example 1(2), wherein an N-terminal portion region (1st to 327th amino acid residue) or a C-terminal portion region (316th to 648th

amino acid residue) of the c-Raf1 is fused with the Flag peptide, and the expression vector of each S-JSAP1 obtained in Example 1(2) using TransIT-LT1 (manufactured by Mirus), and then the COS-7 cells were cultured to transiently express the polypeptide derived from each of the introduced vectors.

In this case, since the expression of full length c-Raf1 in COS-7 cells was considerably low, Raf-N and Raf-C as partial sequences of c-Raf1 were used in this experiment.

The results are shown in Fig. 20. The Raf-C bound to JSAP1 (lane 4 in Fig. 20), but Raf-N did not bind (lane 2 in Fig. 20). Also, the binding affinity of Raf-C was lower than the affinity of MEKK1 (lanes 4 and 6 in Fig. 20).

It was found from the above that JSAP1-binding regions of MAPKKK (MEKK1), MAPK (SEK1 and MKK7) and MAPK (JNK3) related to the JNK3 pathway are different from each other.

In the case of JSAP1a, the binding region for MEKK1 was the 343rd to 1053rd amino acid residue region, the binding region for SEK1 and MKK7 was the 1054th to 1305th residue region, and the binding region for JNK3 was a 115th to 274th residue region.

It was considered that JSAP1 has a leucine-zipper structure. Amino acid sequence numbers of leucine residues constituting the leucine-zipper structure in each JSAP1 are shown below.

JSAP1a: 392, 399, 406, 413, 420, 427

JSAP1b: 401, 408, 415, 422, 429, 436

JSAP1c: 424, 431, 438, 445, 452, 459

JSAP1d: 423, 430, 437, 444, 451, 458

Based on the above, it was considered that each JSAP1 is functioning by existing as a homo or hetero dimer.

#### 5) Analysis of function of JSAP1 as scaffold protein in JNK3 pathway using reporter system

The activation of the JNK3 pathway by over-expression of full length JSAP1 was analyzed.

5XGAL4-LUC reporter expression vector (manufactured by Stratagene), GAL4-c-Jun expression vector (1st to 223rd residues containing a c-Jun activation domain, manufactured by Stratagene) and RL control vector (manufactured by Promega) were introduced into P19 cells differentiated by retinoic acid, originally expressing JSAP1a and JNK3.

Into the P19 cells, full length S-JSAP1a-FL expression vector or S-JSAP1a-Δ5 (1st to 343rd residues) expression vector and/or constitutively activated S-Cdc42 (G12V) expression vector were introduced.

The P19 cells were cultured to transiently express the polypeptide derived from each of the introduced vectors.

After culturing for 24 hours, the luciferase activity was measured, and the GAL4-c-Jun transcription activity, namely JNK3 activity, was calculated. The

relative value of the luciferase activity was obtained by correcting it with the activity value of RL luciferase.

The results are shown in Fig. 21.

Cdc42 (G12V) increased the JNK3 activity, and over-expression of JSAP1a increased the JNK3 activity to a similar level of that of Cdc42 (G12V).

When cells in which Cdc42 (G12V) and JSAP1a were simultaneously expressed, the JNK activity was increased additively to the case of single use.

On the other hand, the JNK3 activity was inhibited when JSAP1a-Δ5 (1st to 343rd amino acid residues) and Cdc42 (G12V) were simultaneously expressed.

The influence of over-expression of full length JSAP1 upon the ERK pathway was examined in the same manner as the above.

5XGAL4-LUC reporter expression vector, GAL4-Elk1 (307th to 427th residues containing an Elk1 activation domain) expression vector and RL control vector were introduced into P19 cells differentiated by retinoic acid.

Into the P19 cells were introduced S-JSAP1a-FL (full length JSAP1a) expression vector and/or the expression vector of constitutively activated ΔRaf1 (*Mol. Cell. Biol.*, 9: 639 (1989)) polypeptide with Flag (Flag-ΔRaf1).



The P19 cells were cultured for transient expression of the polypeptide derived from each of the introduced vectors.

The luciferase activity was measured after culturing for 24 hours, and the GAL4-Elk1 transcription activity, namely the ERK activity, was calculated. The relative value of the luciferase activity was obtained by correcting it with the activity value of RL luciferase.

The results are shown in Fig. 22.

It was revealed that over-expression of JSAP1a inhibits the ERK activity.

The JSAP1a bound to all of the MAPKKK, MAPKK and JNK3 existing in the JNK3 pathway, and it was concluded from the above results that it functions as an important scaffold polypeptide which effectively and specifically activate the JNK3 pathway.

6) Construction of system for screening inhibitor which inhibits phosphorylation of JSAP1 by JNK3

A fusion protein of a partial protein corresponding to the 114th to 274th positions of the amino acid sequence of JSAP1a (containing the JNK3 binding region and three Thr residues to be phosphorylated) with GST was prepared as shown below, to be used as the phosphorylation substrate of JNK3.

A forward primer having the nucleotide sequence represented by SEQ ID NO:17 and a reverse primer having the nucleotide sequence represented by SEQ ID NO:18 were synthesized as the JSAP1a-specific PCR primers.

The forward primer has a nucleotide sequence in which an *EcoRV* recognition sequence is introduced into just before a nucleotide sequence corresponding to a region of the 446th to 464th positions in the nucleotide sequence of cDNA encoding the JSAP1a having the nucleotide sequence represented by SEQ ID NO:1.

The reverse primer has a nucleotide sequence in which a stop codon is introduced into just after a nucleotide sequence corresponding to a complementary sequence of a region of the 909th to 928th positions in the nucleotide sequence of cDNA encoding the JSAP1a having the nucleotide sequence represented by SEQ ID NO:1, and also an *EcoRI* recognition sequence is introduced further just thereafter.

PCR by KOD DNA polymerase (manufactured by Toyobo) was carried out using these two kinds of primers and, as the template, the expression plasmid pET32a containing full length JSAP1a prepared in Example 1(2) by Thermal cycler 450 (manufactured by Takara).

The thus amplified JSAP1a cDNA fragment was separated by agarose gel electrophoresis, and the fragment

was extracted using Qiaex II DNA Extraction Kit (manufactured by Quiagen).

In the same manner, a DNA fragment of pBluescript II KS<sup>-</sup> digested with restriction enzymes *EcoRI* and *EcoRV* was extracted.

The resulting linear chain DNA fragment of pBluescript II KS<sup>-</sup> and JSAP1a DNA fragment were connected by carrying out the ligation reaction at 16°C using DNA Ligation Kit (manufactured by Takara) to obtain plasmid pRH1001 in which the JSAP1a DNA fragment is inserted into pBluescript II KS<sup>-</sup>.

Competent cells of *E. coli* were transformed with the pRH1001, and then the *E. coli* was cultured overnight on LB plate medium (1% tryptone, 0.5% yeast extract, 1% NaCl) to which ampicillin had been added.

*E. coli* obtained from the thus formed colony was cultured in 2 ml of TB medium (a medium prepared by adding 900 ml of water to 12 g of tryptone, 24 g of yeast extract and 4 ml of glycerol, sterilizing the mixture using an autoclave, cooling it to 60°C and then adding thereto 100 ml of a sterilized potassium phosphate solution (0.17 M KH<sub>2</sub>PO<sub>4</sub> and 0.72 M K<sub>2</sub>HPO<sub>4</sub>)) to which ampicillin had been added, and pRH1001 was extracted from the *E. coli*.

By digesting the pRH1001 with *EcoRI* and *EcoRV*, and determining nucleotide sequence of the resulting DNA

fragment, it was confirmed that it coincides with the nucleotide sequence encoding JSAP1a.

A DNA fragment of pRH1001 digested with *EcoRI* and *EcoRV* was separated by an agarose gel electrophoresis, and the fragment was extracted using Qiaex II DNA Extraction Kit (manufactured by Quiagen).

In the same manner, a DNA fragment of pGEX-3X (manufactured by Pharmacia) digested with restriction enzymes *EcoRI* and *SmaI* was extracted.

The resulting linear chain DNA fragment of pGEX-3X and pRH1001 DNA fragment were connected by carrying out the ligation reaction at 16°C using DNA Ligation Kit (manufactured by Takara) to obtain plasmid pRH1003 in which the JSAP1a DNA fragment is inserted into pBluescript II KS<sup>-</sup>.

Competent cells of *E. coli* were transformed with the pRH1003, and then the *E. coli* was cultured overnight on LB plate medium to which ampicillin had been added.

*E. coli* obtained from the thus formed colony was cultured in 2 ml of TB medium to which ampicillin had been added, and pRH1003 was extracted from the *E. coli*.

The pRH1003 is *E. coli* expression vector having a DNA fragment encoding a GST-JSAP1 fusion protein.

The above *E. coli* having pRH1003 was pre-cultured at 37°C overnight in 20 ml of TB medium to which ampicillin had been added.

The resulting culture was added to 440 ml of TB medium to which ampicillin had been added, and cultured at 25°C for 5 hours, and then isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was added to the culture, followed by culturing at 25°C for 15 hours.

Bacterial cells were recovered from the resulting culture by centrifugation. To the cells, 20 ml of PBS containing 0.1 mg/ml of lysozyme was added and incubated at 4°C for 1 hour.

After incubation, 200  $\mu$ l of 10% N-lauroylsarcosine was added thereto and the bacterial cells were disrupted using a sonicator.

The thus disrupted bacterial cells were centrifuged at 100,000  $\times$  g and 4°C for 1 hour to obtain a cytoplasm fraction.

The GST-JSAP1 fusion protein was purified from the cytoplasm fraction by the following method using Glutathione Sepharose 4B (manufactured by Pharmacia)

That is, 5 ml of gel of the Glutathione Sepharose 4B was packed in a column and equilibrated with PBS containing 0.1% N-lauroylsarcosine. The equilibrated Glutathione Sepharose 4B was allowed to adsorb GST-JSAP1 fusion protein and washed with 30 ml of PBS containing N-lauroylsarcosine, and then the GST-JSAP1 fusion protein was eluted with 20 ml of an elution buffer (PBS containing 5 mM reduced form glutathione and 0.1% N-lauroylsarcosine). The

eluate was dialyzed against PBS to obtain a purified GST-JSAP1 fusion protein solution.

Since the purified GST-JSAP1 fusion protein solution can be preserved at  $-80^{\circ}\text{C}$ , it was used by thawing it prior to use.

$\Delta\text{MEKK1}$ , JNK3 and activated JNK3 were prepared and obtained as shown below.

COS-7 cells were inoculated into a 60 mm plate at a  $2 \times 10^5$  to  $5 \times 10^5$  cells/ml DMEM, followed by culturing overnight at  $37^{\circ}\text{C}$ .

To 300  $\mu\text{l}$  of OPTI-MEM (Gibco BRL, manufactured by Life Technologies), 9  $\mu\text{l}$  of FuGene 6 transfection reagent (manufactured by Hoffmann-La Roche) was added, and to the resulting solution, 0.1 to 0.5  $\mu\text{g}$  of the expression vector inserted with cDNA encoding  $\Delta\text{MEKK1}$  and/or 4.5 to 4.9  $\mu\text{g}$  of the expression vector inserted with cDNA encoding JNK3, prepared in Example 1(2), were added and incubated at room temperature for 15 minutes.

The resulting mixture solution was added slowly by three drop portions to the above COS-7 cells and mixed, and then the cells were cultured at  $37^{\circ}\text{C}$  for 30 to 40 hours.

The cells were recovered using a scraper and washed with 10 ml of PBS.

To the resulting COS-7 cells, 1 ml of a cell lysis buffer (50 mM HEPES/NaOH (pH 7.6), 150 mM NaCl, 0.3% (V/V) Nonidet P-40, 20 mM  $\text{MgCl}_2$ , 1 mM ethyleneglycol bis( $\beta$ -

aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), 20 mM  $\beta$ -glycerophosphate, 10 mM  $\text{Na}_2\text{VO}_4$ , 10 mM NaF, 40  $\mu\text{g/ml}$  phenylmethylsulfonyl fluoride (PMSF), 1  $\mu\text{g/ml}$  pepstatin A, 1  $\mu\text{g/ml}$  leupeptin, 1  $\mu\text{g/ml}$  chymostatin, 2 mM dithiothreitol (DTT)) was added to disrupt the cells.

The resulting cell lysate was centrifuged at 20,000  $\times g$  and 4°C to obtain a supernatant. The supernatant of cell lysate derived from cells introduced with an expression vector into which cDNA encoding  $\Delta\text{MEKK1}$  had been inserted was used as a  $\Delta\text{MEKK1}$  enzyme solution, the supernatant of cell lysate derived from an expression vector into which cDNA encoding JNK3 had been introduced was used as a JNK3 enzyme solution and the supernatant of cell lysate derived from cells introduced with two kinds of expression vectors into which cDNA encoding  $\Delta\text{MEKK1}$  and JNK3 had been inserted was used as an activated JNK3 enzyme solution.

A system for screening an inhibitor which inhibits phosphorylation of JSAP1 by activated JNK3 is described below.

After the reaction in homogenous solution using activated JNK3,  $[\gamma\text{-}^{33}\text{P}]\text{-ATP}$  (74 TBq/mmol, manufactured by New England Nuclear) and GST-JSAP1, the phosphorylation of JSAP1 by activated JNK3 was measured using, as an index, the radioactivity of  $^{33}\text{P}$  incorporated into GST-JSAP1.

A reaction solution for screening ( $3\ \mu\text{M}$  [ $\gamma\text{-}^{33}\text{P}$ ]-ATP (28 kBq/ml),  $2.3\ \mu\text{M}$  GST-JSAP1,  $20\ \text{mM}$   $\text{MgCl}_2$ ,  $20\ \text{mM}$   $\beta$ -glycerophosphate,  $20\ \text{mM}$  p-nitrophenyl phosphate,  $1\ \text{mM}$   $\text{Na}_3\text{VO}_4$ ,  $1\ \text{mM}$  NaF,  $2\ \text{mM}$  DTT, 1% DMSO or a test compound containing 1% DMSO, 2% (V/V) of any one of the three kinds of enzyme solutions prepared in the above (the activated JNK3 enzyme solution is used in the actual screening),  $50\ \text{mM}$  HEPES/NaOH (pH 7.6)) was dispensed in  $100\ \mu\text{l}$  portions into each well of a 96 well plate (manufactured by Corning) incubated at  $30^\circ\text{C}$  for 10 to 30 minutes.

After incubation,  $100\ \mu\text{l}$  of ethanol was added to the reaction solution to terminate the reaction and to precipitate GST-JSAP1.

The precipitate was filtered and adsorbed on UniFilter Plate GF/B (manufactured by Packard) using FilterMate Harvester (manufactured by Packard).

The plate was washed at least three times with PBS and dried, and then  $40\ \mu\text{l}$  of a scintillator Microscint-20 (manufactured by Packard) was added thereto to measure the radioactivity using a detector TopCount-HTS (manufactured by Packard).

The results are shown in Table 1.



Table 1 Phosphorylation of GST-JSAP1 by COS-7 lysate

Enzyme solution * <sup>1</sup>	Phosphorylation activity (pmol/mg/min)
-	23
ΔMEKK1	26
JNK3	37
Activated JNK3	978

\*<sup>1</sup>: Enzyme solution used at the time of preparation of the reaction solution for screening

In the above system, GST-JSAP1 was specifically phosphorylated only when the activated JNK3 enzyme solution was used in the reaction solution for screening.

Accordingly, the above system in which the activated JNK3 enzyme solution was used in the reaction solution for screening has a possibility of being used as a system which can efficiently screen an inhibitor that inhibits phosphorylation of JSAP1 by JNK3. The true phosphorylation activity can be calculated by subtracting the phosphorylation activity in a system which does not use the activated JNK3 enzyme solution.

The fact that the above activated JNK3 enzyme solution is a solution containing the activated JNK3 was confirmed by the following experiment.

Using 10 μg (as the amount of enzyme) of each of the ΔMEKK1 enzyme solution, JNK3 enzyme solution and activated JNK3 enzyme solution prepared above, SDS-PAGE was carried out and each of the electrophoresed proteins was

transferred on membrane Hybond-ECL (manufactured by Amersham-Pharmacia Biotech).

Western blotting was carried out using the membrane, and anti-active JNK polyclonal antibody (manufactured by Promega) and anti-Flag M2 monoclonal antibody (manufactured by Sigma) as probes. Development of color was carried out using a detection buffer (100 mM Tris/HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.33 mg/ml nitro blue tetrazolium (NBT), 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), p-toluidine salt). The results are shown in Fig. 23.

The band of phosphorylated activated JNK3 was observed only in the activated JNK3 enzyme solution (lane 2 in Fig. 23A). The band of JNK3 protein was observed only in the JNK3 enzyme solution (lane 3 in Fig. 23B), and band shift of JNK3 by phosphorylation was observed (lanes 2 and 3 in Fig. 23B).

K<sub>m</sub> values of ATP and GST-JSAP1 as the substrates for phosphorylation reaction in the screening system constructed above were calculated according to the method described in *Introduction to Enzyme Kinetics, Biochemical Experimentation*, 21, edited by Masatake Ohnishi, Japan Scientific Societies Press (1987).

Both of ATP and GST-JSAP1 showed Michaelis-Menten substrate-reaction initial rate curve, and it was found by Lineweaver-Burk plot that K<sub>m</sub> values of ATP and GST-JSAP1 were 6.3  $\mu$ M and 0.48  $\mu$ M, respectively.

### Example 3 Binding of JSAP3 to JNK3

COS-7 cells were transfected with the S-JSAP3 (full length) expression vector and the Flag-JNK3 expression vector, prepared in Example 1(2), using TransIT-LT1 (manufactured by Mirus), and then the COS-7 cells were cultured to transiently express the polypeptide derived from each of the introduced vectors.

After culturing for 34 hours, the cells were dissolved in buffer B, S Protein Agarose was added thereto, and then S-JSAP3 and polypeptide capable of binding to S-JSAP3 were precipitated.

The resulting fraction was separated by SDS-PAGE and transferred on membrane Immobilon-P (manufactured by Millipore).

Western blotting was carried out using the membrane and anti-Flag M5 monoclonal antibody (manufactured by Kodak) as the probe, and the polypeptide capable of binding to the antibody (Flag-JNK3) was visualized using ECL detection system (manufactured by Amersham).

The results are shown in Fig. 24.

It was confirmed that JSAP3 binds to JNK3 (lane 2 in Fig. 24).

Binding of JSAP3 to a transcription factor ATF2 and the ATF2 binding region were analyzed by the following method.

According to the method described in Example 1(2), JSAP3 (full length polypeptide), Trx•S, Trx•S-ATF2 (1st to 107th amino acid residues) and Trx•S-ATF2 (1st to 116th amino acid residues) fusion polypeptide were expressed in *E. coli* and then recovered by allowing them to bind to S Protein Agarose.

<sup>35</sup>S-labeled form of S-JSAP3 was prepared using the S-JSAP3 (full length) expression vector obtained in Example 1(2) by *in vitro* translation with TNT T7 Quick Coupled Transcription/Translation System (manufactured by Promega).

The resulting <sup>35</sup>S-labeled form of S-JSAP3 and each of Trx•S, Trx•S-ATF2 (1st to 107th amino acid residues) and Trx•S-ATF2 (1st to 116th amino acid residues) were mixed in buffer A, and the reaction was carried out at 4°C for 2 hours while stirring the reaction solution by rotating its container tube.

After the reaction, the reactant was washed three times with buffer A, and the resulting precipitate was separated by SDS-PAGE and analyzed by autoradiography.

The results are shown in Fig. 25.

It was found that JSAP3 binds to a region of 108th to 116th amino acid residues of ATF2.

Among the JSAP3-binding ATF2 region of 108th to 116th residues, a sequence of the 108th to 112th residues completely coincided with an already reported CtBP binding sequence motif PLDLS (*J. Biol. Chem.*, 273: 8549 (1998)).

#### Example 4 JSAP4

##### 1) Binding of JSAP4 to JNK3

COS-7 cells were transfected with the S-JSAP4 (full length) expression vector and the Flag-JNK3 expression vector, prepared in Example 1(2), using TransIT-LT1 (manufactured by Mirus), and then the COS-7 cells were cultured to transiently express the polypeptide derived from each of the introduced vectors.

After culturing for 34 hours, the cells were dissolved in buffer B, S Protein Agarose was added thereto, and then S-JSAP4 and polypeptide capable of binding to S-JSAP4 were precipitated.

The resulting fraction was separated by SDS-PAGE and transferred on membrane Immobilon-P (manufactured by Millipore).

Western blotting was carried out using the membrane and anti-Flag M5 monoclonal antibody (manufactured by Kodak) as the probe, and the polypeptide capable of binding to the antibody (Flag-JNK3) was visualized using ECL detection system (manufactured by Amersham).

The results are shown in Fig. 24.

It was confirmed that JSAP4 binds to JNK3 (lane 4 in Fig. 24).

The JNK3 binding region of JSAP4 was analyzed by the following method.

The GST or GST-JNK3 fusion protein expression vector obtained in Example 1(2) was expressed in *E. coli*, and the protein was adsorbed to Glutathione-Agarose (manufactured by Sigma).

Using the S-JSAP4 expression vector of full length or partial length JSAP4 obtained in Example 1(2), <sup>35</sup>S-labeled form of various S-JSAP4 preparations were prepared by *in vitro* translation using TNT T7 Quick Coupled Transcription/Translation System (manufactured by Promega), separated by SDS-PAGE and then analyzed by autoradiography.

The results are shown in Fig. 26.

The resulting <sup>35</sup>S-labeled form of each of various S-JSAP4 preparations and the GST or GST-JNK3 fusion protein adsorbed on Glutathione-Agarose were put into a tube containing buffer A and mixed while rotating the tube, and then the mixture was incubated at 4°C for 2 hours.

After incubation, the reactant was washed three times with buffer A, and the protein adsorbed to Glutathione-Agarose was separated by SDS-PAGE and analyzed by autoradiography.

The results are shown in Fig. 27.

It was found that JNK3 binds to a region of the 1063rd to 1331st amino acid residues of JSAP4.

## 2) Binding of JSAP4 to JNK1 and JNK2

COS-7 cells were co-transfected with the Myc-tag-added Myc-JSAP4 (1063rd to 1331st amino acid residue region; a region which binds to JNK3) expression vector and the His-S-tag-added His-S-JNK1, His-S-JNK2, His-S-JNK3 or His-S-ERK2 expression vector, prepared in Example 1(2), was carried out using FuGene 6 transfection reagent (manufactured by F. Hoffmann-La Roche).

The COS-7 cells were cultured to transiently express the polypeptide derived from each of the introduced vectors.

After culturing for 34 hours, the cells were dissolved in buffer B and immunoprecipitated using S-protein agarose (manufactured by Novagen).

The resulting precipitation fraction was separated by SDS-PAGE and transferred on membrane Immobilon-P (manufactured by Millipore).

Western blotting was carried out using the membrane and anti-Myc monoclonal antibody 9E10 (manufactured by Boehringer Mannheim) as the probe, and the Myc-JSAP4 capable of binding to the antibody was visualized using ECL detection system (manufactured by Amersham). Also, the expression of Myc-JSAP4 in respective co-transfected cells was confirmed by Western blotting using anti-Myc monoclonal antibody 9E10 (manufactured by Boehringer Mannheim), and the expression of His-S-JNK1, His-S-JNK2, His-S-JNK3 and

His-S-ERK2 was confirmed by Western blotting using anti-His polyclonal antibody (manufactured by Santa Cruz) after the cells were lysed.

The results are shown in Fig. 28.

It was revealed that JSAP4 binds to JNK1, JNK2 and JNK3, whereas it does not bind to ERK2.

### 3) Expression analysis of JSAP4 mRNA by Northern hybridization

Northern hybridization was carried out according to the method described in *Proc. Natl. Acad. Sci. USA*, 92: 4972 (1995).

Specifically, each of mouse testis, large intestine, heart, lung, kidney, brain, spleen and liver was analyzed using <sup>32</sup>P-labeled JSAP4 and  $\beta$ -actin cDNA probes.

The results are shown in Fig. 29.

Expression of JSAP4 was found slightly in the testis, heart and kidney, and particularly, its most abundant expression was found in the brain.

Since a sequence called WD40-repeat was found in JSAP4, it was expected that the sequence could be concerned in an interaction (binding) with other protein, and it was expected that it would take part in the JNK3 pathway on its signal transduction (*FEBS Lett.*, 307: 131 (1994)).



#### 4) Function analysis of JSAP4 in JNK3 pathway using reporter system

Full length JSAP4 was over-expressed, and influence thereof on the JNK3 pathway was analyzed by the following method.

5XGAL4-LUC reporter expression vector (manufactured by Stratagene), GAL4-c-Jun expression vector (1st to 223rd residues containing a c-Jun activation domain, manufactured by Stratagene) and RL control vector (manufactured by Promega) were introduced into COS-7 cells using FuGENE6 transfection reagent (manufactured by F. Hoffmann-La Roche).

At least one of the Myc-JSAP4-FL expression vector, His-S-MEKK1 expression vector and Flag-TAK1 expression vector prepared in Example 1(2) was introduced into the COS-7 cells using the FuGENE6 transfection reagent.

The COS-7 cells were cultured to transiently express the polypeptide derived from each of the introduced vectors.

The luciferase activity was measured after culturing for 34 hours, and the GAL4-c-Jun transcription activity, namely JNK3 activity, was calculated. The relative value of the luciferase activity was obtained by correcting it with the activity value of RL luciferase.

The results are shown in Fig. 30.

Increase in the JNK activity was slight by the JSAP4 per se, whereas the MEKK1 and TAK1 as members of

MAPKKK increased the JNK activity 3 times and 3.2 times, respectively. However, the activation of JNK by MEKK1 and TAK1 was further 2.7 times and 3 times increased, respectively, by the over-expression of JSAP4.

Influence of the over-expression of full length JSAP4 on the ERK pathway was examined in the same manner as the above.

5XGAL4-LUC reporter expression vector, GAL4-Elk1 (307th to 427th residues containing an Elk1 activation domain) expression vector and RL control vector were introduced into COS-7 cells using the FuGENE6 transfection reagent.

The Myc-JSAP4-FL expression vector and/or the expression vector of continuously activated  $\Delta$ Raf1 (*Mol. Cell. Biol.*, 9: 639 (1989)) polypeptide with Flag (Flag- $\Delta$ Raf1) prepared in Example 1(2) were introduced into the COS-7 cells using the FuGENE6 transfection reagent.

The COS-7 cells were cultured to transiently express the polypeptide derived from each of the introduced vectors.

The luciferase activity was measured after culturing for 34 hours, and the GAL4-Elk1 transcription activity, namely ERK activity, was calculated. The relative value of the luciferase activity was obtained by correcting it with the activity value of RL luciferase.

The results are shown in Fig. 31.

The over-expression of JSAP4 had no influence on the activity of the ERK pathway. Also, it had no influence on the activation of ERK by the activated  $\Delta$ Raf1.

Based on the results of 1) and 2) of Example 4, it was concluded that JSAP4 binds to JNK1, JNK2 and JNK3 and has a function of specifically increasing efficiency of the activation of the JNK pathway.

#### Example 5 Binding of JSAP5 to JNK3

Transfection of COS-7 cells with the S-JSAP5 (partial length) expression vector and Flag-JNK3 expression vector prepared in Example 1(2) was carried out using TransIT-LT1 (manufactured by Mirus), and then the COS-7 cells were cultured for transient expression of the polypeptide derived from each of the introduced vectors.

After 34 hours of the culturing, the cells were dissolved in buffer B, S Protein Agarose was added thereto, and then S-JSAP5 and polypeptide capable of binding to S-JSAP5 were precipitated.

The resulting fraction was separated by SDS-PAGE and transferred on membrane Immobilon-P (manufactured by Millipore).

Western blotting was carried out using the membrane and anti-Flag M5 monoclonal antibody (manufactured by Kodak) as the probe, and the polypeptide capable of binding

to the antibody (Flag-JNK3) was visualized using ECL detection system (manufactured by Amersham).

The results are shown in Fig. 24.

It was confirmed that JSAP5 binds to JNK3 (lane 6 of Fig. 24).

#### INDUSTRIAL APPLICABILITY

Neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis and the like, amyotrophic diseases, such as amyotrophic lateral sclerosis and the like, ischemic diseases, brain damage due to stroke, schizophrenia, depression, epilepsy, and various immunological and inflammatory diseases can be prevented and treated using the DNA of the novel polypeptide having a JNK3 binding activity obtained in the present invention.

#### FREE TEXT OF SEQUENCE LISTINGS:

SEQ ID NO:17-Explanation of artificial sequence: Synthetic DNA

SEQ ID NO:18-Explanation of artificial sequence: Synthetic DNA